$P16^{INK4a}$/KI-67 IMMUNOCYTOCHEMISTRY IN IMPROVING THE PREDICTIVE VALUE FOR HIGH GRADE CERVICAL INTRAEPITHELIAL (≥ CIN2) LESIONS IN PAP SMEAR.

A DISSERTATION SUBMITTED IN PART FULFILLMENT OF THE REGULATION FOR THE AWARD OF THE DEGREE OF M.D. PATHOLOGY BRANCH III.

THE TAMIL NADU DR. M.G.R. UNIVERSITY

CHENNAI, TAMIL NADU

May-2018

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CERTIFICATE

This is to certify that this dissertation "$P16^{INK4a}$/ki67 immunocytochemistry in improving the predictive value for high grade cervical intraepithelial (≥ CIN2) lesions in pap smear'' is the bonafide work done by Dr. Vinoth Kumar.G, in part fulfillment of the rules and regulations for the M.D. Branch III (Pathology) Degree Examination of Tamil Nadu Dr.M.G.R. Medical university, to be held in May 2018.

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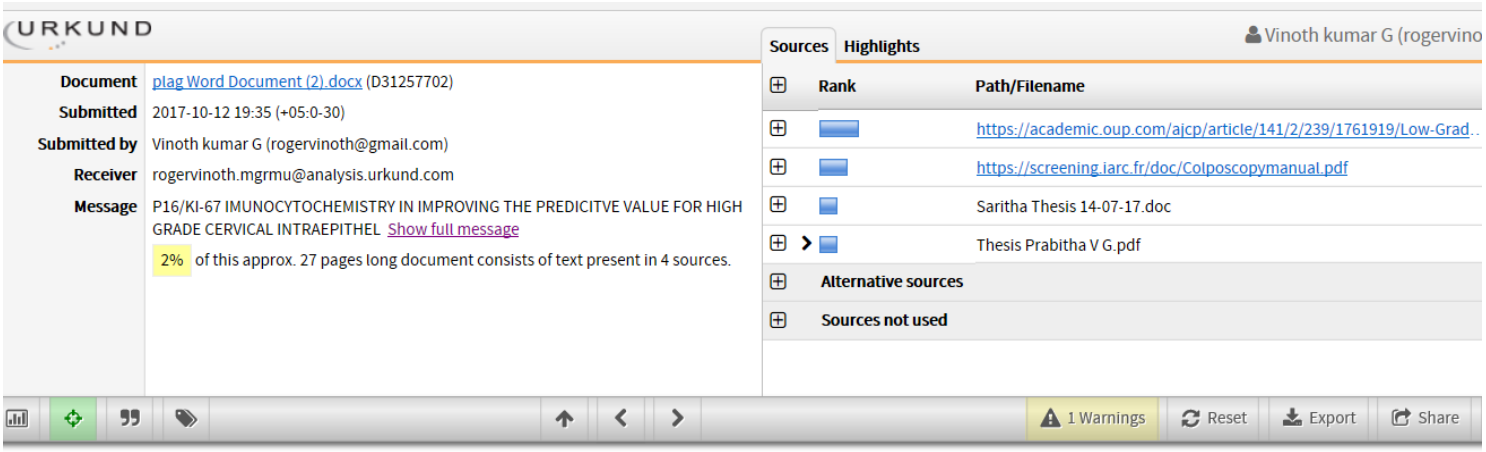

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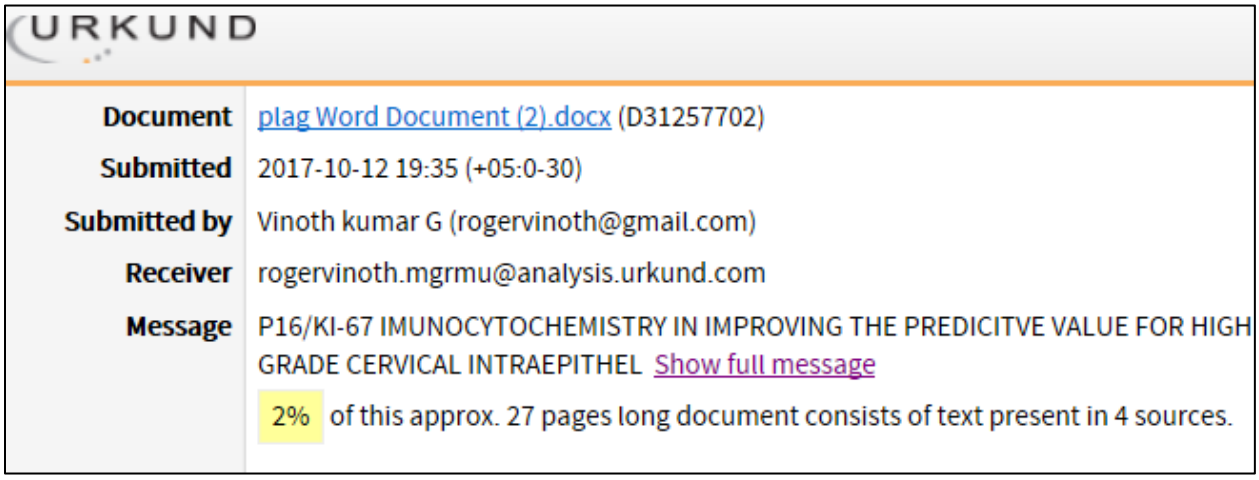

CERTIFICATE

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ACKNOWLEDGEMENT

I would like to thank the Almighty, my family and friends for their help in pursuing my thesis.

I express my warm thanks to Dr. Anne Jennifer Prabhu (General Pathology), Dr. Abraham Peedicayil (Department of Gynaecologic Oncology) and Dr. Priya Abraham (Department of Virology) for their guidance and persistent help for the completion of this dissertation.

I would also like extend my thanks to Dr. Ajith Sebastian, Dr. Priya, Dr. Sandeepan from department of Gynaecologic Oncology for helping me in collecting study samples and all the staff nurses from gynaecology OPD for helping me to collect consent forms.

I would extend my sincere thanks to Mrs. Rachel Evelyn and all cytology staff for helping me to collect thin prep samples, Mr. Doltan from Roche MTM Laboratories for helping me to complete p16/ki-67 immunocytochemistry, Dr. Raghavendran, department of Virology for helping me with the hr-HPV PCR techniques and DR.J.P. Muliyil and Dr. Visalakshi for statistical analysis.

ABBREVIATIONS

ASC-US - Atypical squamous cells - of undetermined significance

ASC-H - Atypical squamous cells - cannot exclude HSIL

LSIL - Low-grade squamous intraepithelial lesion

HSIL - High-grade squamous intraepithelial lesion

SCC - Squamous cell carcinoma

CIN -Cervical intraepithelial neoplasia

hr-HPV - high risk - Human papilloma virus

ICC - Immunocytochemistry

HC-2 - Hybrid capture-2

RLU - Relative lights unit

PPV - Positive predictive value

NPV - Negative predictive value

ASCCP - American Society for Colposcopy and Cervical Pathology

Contents

INTRODUCTION

Cervical cancer remains an important problem, most importantly in developing countries where it is associated with high incidence and increased mortality. It has a potential for prevention with effective screening program. Cervical cancer is the fourth most common cancer in women, and the seventh overall, with an estimated 528,000 new cases in 2012(1).Based on report by Indian council of Medical Research, 20-35 per 1,00,000 women were affected by cervical cancer in India between age group of 33 to 65 years, whereas in developed countries it varies from 1 to 8 cases per 1,00,000 women.

Pap smear is considered as one of the best screening tests in medicine and it has reduced the incidence of cervical cancer significantly to more than 50% (2). However, the limitation of Pap test is its wide range of sensitivity to detect cervical precancerous lesions. Infection with high-risk human papillomaviruses (hr-HPV) for longer period of time has been identified as the important causal factor for developing high-grade cervical intraepithelial neoplasia (CIN) and increase the chance for progression to cervical carcinoma.

Testing for the hr- HPV types is referred to as HPV DNA testing. This test is being increasingly used in screening cervical cancer and can be performed from cytology samples. These tests are suggested for women more than 30 years and diagnosed with ASC-US and LSIL by Pap smear. But HPV DNA testing is not suggested in women less than 30 years due to the more number of HPV infections in this age group. These infections are of high transient nature. Therefore, to improve the screening process,

current standard protocol being followed in the Gynecology outpatient department of CMC is to test for HPV DNA if cervical cytology is suggestive of ASC-US or LSIL. This further directs the clinician to recommend colposcopy.

hr-HPV DNA tests increase the identification of number of women with high grade lesions. This testing has improved sensitivity compare to cervical cytology but has lower specificity. A Few host cell biomarkers were assessed to improve the specificity of cervical screening(3). P16INK4A is one such host cell biomarker which has been recently identified. This is also called as P16 which is a cyclin dependent kinase inhibitor. This is used as a cellular protein marker(4).

Combining p16 with ki-67 proliferation marker, detects transformed HPV cells which are undergoing uncontrolled proliferation. Many studies have assessed the clinical utility p16/ki-67dual immunocytochemistry for detecting of high grade (≥ CIN 2) lesions(5). The purpose of this study is to assess the significance of p16/ki67 immunocytochemistry in improving the efficiency of the screening system for cervical cancer.

AIMS AND OBJECTIVES

AIM:

- To assess the significance of $P16^{INK4a}$/ki67 immunocytochemistry in improving the predictive value for high grade cervical intraepithelial (≥ CIN2) lesions on Pap smears.

Objectives:

- To identify 100 consecutive cases of ASC-US/LSIL/ASC-H/HSIL on thin prep pap smears in women aged more than 25 years.

- To perform, interpret and correlate the results of p16/Ki67immunocytochemistry on these thin prep cervical smears with histopathological features and high-risk HPV DNA.

REVIEW OF LITERATURE

The earliest description regarding cervical intraepithelial pre-cancerous lesion was given by Sir John Williams in 1888(6). Originally the term carcinoma in situ was coined by Broders to describe lesions on cervical biopsies that was composed of cells that morphologically similar to carcinoma, but without invasion of the basement membrane(7).

However, by the early 1950s, it became evident that there were surface lesions that had abnormal histopathological features which did not fulfil the carcinoma in situ criteria. These lesions had less risk for subsequent development for cancer**.** In 1952, Reagen and Hicks coined the term 'atypical hyperplasia' to describe these histopathological lesions. However in the subsequent year, they replaced this term with dysplasia, which was further graded as mild, moderate and severe(8). Albeit, the most profound change in cervical histopathological terminology came about in 1969, when Richart proposed that carcinogenesis in the cervix was a continuum of disease that begins as mild dysplasia and progresses through mild, moderate, and severe dysplasia to carcinoma in situ. He therefore proposed that the artificial terminological distinction between dysplasia and carcinoma in situ be abandoned and coined the term 'cervical intraepithelial neoplasia' to emphasize its association with cancer(9).

The CIN terminology became the most widely accepted histopathological terminology for cervical cancer precursors in the late 1970s. The CIN terminology divided precancerous cervical lesion into 3 groups: CIN 1 corresponds to mild dysplasia, CIN 2 corresponds to moderate dysplasia, and CIN 3 to both severe dysplasia and carcinoma in situ. At the onset of this classification, CIN was thought to define a spectrum of cervical

precancerous lesions, which shared common etiology and histological changes. Infection with high-risk types of HPV plays an important etiology for the development of cervical cancer. Thus, a new model of cervical carcinogenesis has been developed. This model has three discrete steps: Initial infection with a high-risk type of HPV, progression to a histologically defined precursor lesion that requires persistence of the HPV infection, and invasion(10,11).

Based on above model, the spectrum of histological changes that are referred to as CIN do not represent a single disease process at different stages in its development but instead two distinct biological entities, one a productive viral infection and the other a true neoplastic process confined to the epithelium(12). The screening test for cervical cancer in the last 70 odd years has been the Papanicolaou test which is also known as Pap smear or Pap test. It was developed by Georgios Papanicolaou in 1940. The cytopathology also had a similar terminology problem before the Bethesda conferences. The previous terminology in cytology was mild, moderate and severe dysplasia. These conferences proposed a new terminology for reporting cervical cytology. Now this is commonly known as The Bethesda System (TBS) which created standard reporting terms and criteria for each interpretive category(13).

This terminology uses the term low-grade squamous intraepithelial lesion (LSIL) for lesions previously categorized as CIN 1/low grade dysplasia and high-grade squamous intraepithelial lesion (HSIL) for lesions previously known as CIN 2/moderate dysplasia and CIN 3/high grade dysplasia(14,15).

Squamous intraepithelial lesion (SIL) with only two gradations (low and high grade) reflects the different biologic states of productive HPV infections versus lesions with a higher risk of transitioning to precancer and ultimately cancer. The Bethesda System also enabled the development of clinical management guidelines linked to these standardized terminologies. The Bethesda System(TBS) for cervical cytology supported a two-tiered classification.

Promotion of a 2-tiered terminology for histology in the 1990s was not supported by professional organizations, hence this terminology for histopathological report was never widely adopted. The 2001 and 2006 American society of colposcopy and cervical pathology (ASCCP) Consensus Guidelines and World Health Organization (WHO) used a 2-tiered terminology for cervical precancerous lesion, except in adolescents and young women with CIN 2 and CIN 3 (16,17). At present, WHO and 2012 ASCCP Consensus Guidelines for the clinical management of cervical histological abnormalities, uses a 2-tiered terminology for precancerous cervical lesions(18) i.e. LSIL and HSIL.

The primary aim in using a 2-tiered system for reporting cervical histology in young women and adolescents is that, there can be expectant management of CIN 2 with the option to follow up, but not for CIN 3 which needs early intervention (18). The 2-tiered system better reflects the association between the biology of HPV with precancerous cervical lesions and also helps in management(19).

Discordance between cytology and histopathology:

In USA, the median reporting rate of ASCUS is 4.7% according to a 2003 survey by College of American Pathology and median reporting rate of ASC-H in 2003 was 0.4%(20). Data obtained from the College of American Pathologists (CAP) show that, in 2006 the median rate for LSIL was 2.5 % and HSIL was 0.5%. They also found that, there is an overall 10–15 % inter- pathologist discordance rate between the diagnosis of LSIL and HSIL interpretations in cervical cytology (21). It was found that, 15–25 % of women with LSIL in cytology are found to have CIN 2+ lesions in histopathology(22).

Etiology:

Major risk factors for cervical cancer are presence of high-risk types of HPV and lack of cervical screening. Factors such as smoking, use of oral contraceptive and immunosuppression may result in increase in the risk for cervical carcinoma in women infected with high-risk types of HPV(23).

Pathogenesis:

In the late 1970s, Dr. Harald Zur Hausen proposed that there might be an association between HPV and precancerous and cancerous cervical lesion(24). A large number of epidemiological studies subsequently linked infection by specific types of HPV to the development of anogenital cancers. Presence of high-risk HPVs play a critical role in the development of most precancerous and cancerous cervical lesions (25).

The human papilloma viruses belong to the genus alpha-papillomavirus. The most important of these are HPV 16, HPV 18 and 45. Papilloma viruses are epitheliotropic and predominantly infect skin and mucous membranes. These produce abnormal epithelial proliferations at the site of HPV infection. These benign epithelial proliferations or papillomas can undergo malignant transformation in certain circumstances. HPV infections occur on the skin, mucous membranes, oral cavity, conjunctiva, larynx, tracheobronchial tree, urinary bladder, esophagus, anus and genital tract. HPVs replicate only in the nucleus of infected cells(25).

More than 40 types of HPV can infect the anogenital tract and based on their associations with anogenital and cervical cancers, 13 different anogenital HPVs have been identified and classified by the International Agency for Research on Cancer(IARC) as oncogenic. These include HPV 16, 18, 31, 33, 35, 39,45, 51, 52, 56, 58, 59 and 66 (25). HPV types 68 and 82 are also considered oncogenic by few others(26). HPV 6 and 11, which are the two types that are most commonly associated with condyloma acuminata, are not implicated in the development of cervical cancer. But they are associated with carcinomas of the larynx, vulva, penis and anus (25).

In LSIL, HPV 16 was found in 26% of HPV positive cases and 8% for HPV 18. HPV 31, 51 and 53 were the other most commonly identified types in LSIL, each being present in approximately 10–12% of the lesions. Multiple HPV types are frequently found in low-grade lesions.

In contrast to LSIL, more than half of HPV types are associated with HSIL. Prevalence of HPV 16 in cases of HSIL, in studies from different regions from the globe range from 30% to 70%(27). HPV types 16 and 18 were also found in 52% of the HSIL lesions. HPV 31, 33, 58 and 52 are the other common types found in HSIL. Multiple types of HPV are less commonly found in HSIL than in LSIL.HPV types 16 and 18 are found in about 70% of invasive cervical cancers worldwide. HPV types 31, 33, 35, and 45 are each found in approximately 3–4% of cervical cancers (27). The other high-risk HPV types are found to be associated only with about 2.5% of cervical cancers and most are found in less than 1%.

Genomic Organization of HPV:

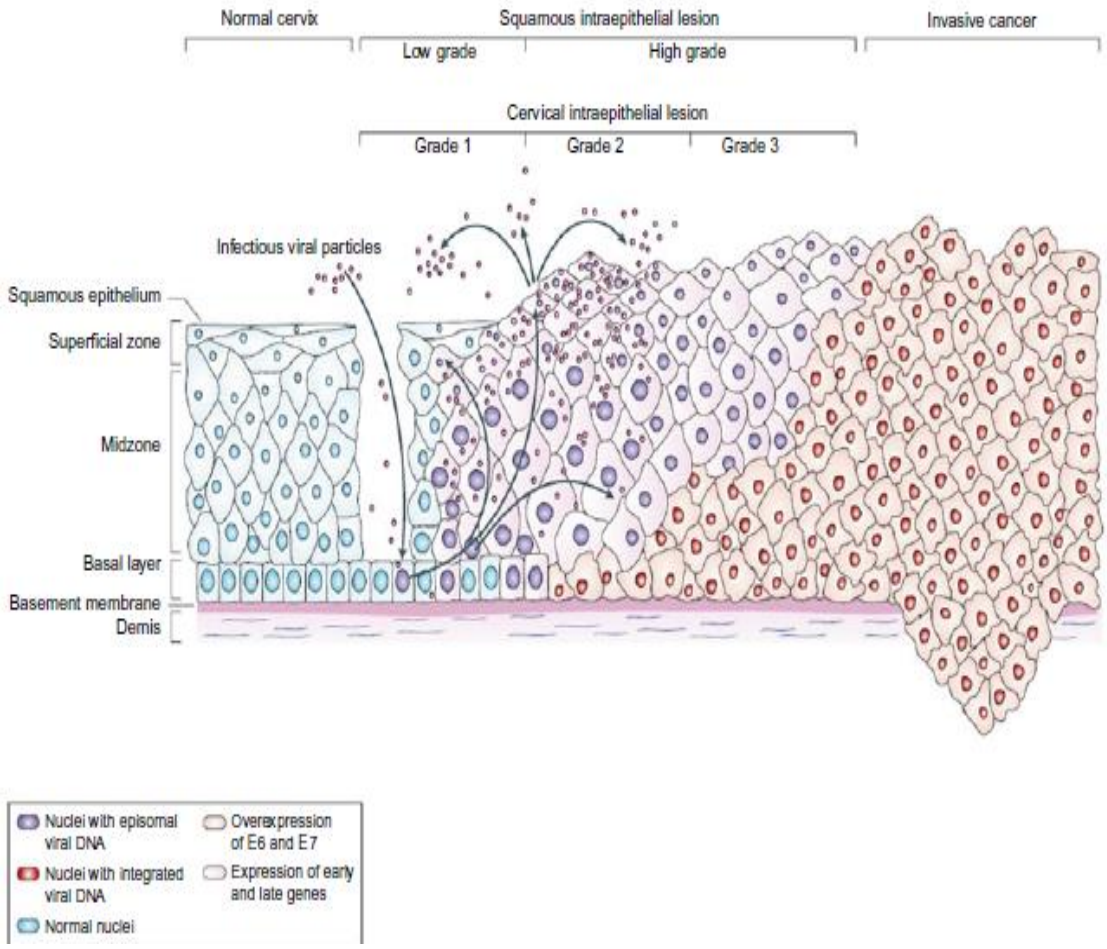


Fig 1: Pathogenesis of HPV in precancerous and cancerous cervical lesion

Adapted from Woodman et al, Nat Rev Cancer 2007;7:11-22

The genomic organization of different types of HPV appear to be similar. The viral genome has three different regions: an upstream regulatory region, the early region and the late region. The LCR is important for viral replication and transcription. The early

region is transcribed early in the viral life cycle and encodes proteins that are needed for viral replication. The late region encodes for viral structural proteins that are produced late in the life cycle. Open reading frames (ORFs) of early region encode proteins needed for viral replication and maintenance in infected cells. The early region of HPV genome contains E5, E6 and E7 transforming regions. The E6 and E7 ORFs encode the major transforming genes of HPV (28). E6 can bind to p53 and result in rapid proteolytic degradation of p53, through ubiquitin-dependent mechanism, thus preventing apoptosis. E7 binds to the retinoblastoma(Rb) gene product and also to ‘‘Rb-like proteins’’. Binding of E7 to Rb blocks the cell proliferation by inhibiting the function of these endogenous tumor suppressors. The final result of over expression of E6 and E7 within infected cells is unrestricted cell proliferation.

The late region of HPV is downstream to the early region and contains L1 and L2 viral capsid proteins. The L1-encoded protein is the major capsid protein. The L2-encoded protein is a minor capsid protein that is much more variable among viral types. Transcription from the L1 and L2 ORFs occurs as a late event in the life cycle of virus at a time when infectious virus is being produced. Viral-like particles (VLPs) composed of L1 capsid proteins have recently been introduced as prophylactic HPV vaccines(29). The initial site of infection is most likely the basal cells where the HPV virus reaches through breaks in the epithelium. Once the virus enters these cells, they can exist in two forms. The forms are non-productive and productive. The non-productive form is when the virus exists in the cell, but does not replicate and produce virions. It is also called as latent

infection. This latent infection does not produce characteristic morphological changes in the epithelial cell. They can only be detected by molecular testing. Productive viral infection occurs independent of host chromosomal DNA synthesis and results in replication of viral DNA in the cervical epithelium. The infected cervical epithelial cells move toward epithelial surface as they mature. Transcriptional factors produced by the epithelium stimulate the production of viral capsid protein. Viral associated effects of HPV produce characteristic cytological and histological changes seen in the cervical epithelial cells due to large amounts of virions. These viral-associated effects include nuclear atypia, multinucleation, acanthosis, cytoplasmic vacuolization and koilocytosis.

Most HPV infections are transient in nature and usually they undergo clearance or may became persistent latent infection within one to two years of detection(30). Cell mediated immunity plays an important role in clearance or development of latent infection. High risk HPV infection will get cleared much slower than low risk HPV infection. The majority of HPV infections undergo clearance or become latent within 1–2 years of detection. Clearance or development of latency is presumed to be mediated by cell-mediated immunity. If the infection is persistent for 36 months, the chance of clearance of HPV infection reduces considerably. Hence, persistent high risk HPV infection for more than two years increases the risk to of progression to a high grade precursor cervical lesion or invasive cervical cancer(31).

Approximately, 10 % of high risk HPV infections will stay for more than two years. In a few women, recurrent HPV infections can occur after the clearance of the previous infection. There is strong association between immunodeficiency and detection of HPV in patients with HIV infection detected in one study(32). The above facts suggest that women who appear to have cleared the HPV infection continue to harbor low copy number of latent HPV in the epithelium. HPV has high prevalence in sexually active population. Prevalence of high risk HPV infection is high in late teens and drops with increasing age, in women with normal cervical cytology(33). High HPV prevalence is seen in less developed countries due to unknown reasons, but this may be related to poor personal hygiene, sexual practices and increase in burden of comorbid conditions in the general population.

Only about one-third of high risk HPV DNA positive women will show abnormal cytology. The overall incidence of abnormal cervical cytology is approximately 25-50% within the first 2 years of infection. The risk of abnormal cytology further declines to baseline level in the general population within 4 years(34). Risk factor for HPV persistence and development of HSIL are not well identified but the type of HPV is important in subsequent development of high grade lesion. Cumulative risk for the development of HSIL in women with persistent infection for 3-5 years due to HPV 16 is approximately 40 %. Multiple types of HPV infections increase the risk for HSIL. It may be due to the sum of the risks for each individual type (35).

Due to understanding of the pathogenesis in squamous intraepithelial lesion, researchers have proposed that LSIL is multicellular in origin whereas HSIL is unicellular in origin. LSIL develops within latently infected cervical epithelial cells and is linked with multiple types of HPV infection. HSIL is frequently aneuploid associated with a single type of HPV and may have integrated HPV DNA(36). Squamous intraepithelial lesions can be monoclonal or polyclonal. Low risk HPV types in LSIL are polyclonal whereas high risk HPV types in LSIL are monoclonal(37). This shows that LSIL associated with low risk HPV types are biologically different from lesions identified as low grade by histology, but harbor high risk HPV types. Studies on CIN1 and CIN2 have shown that 83 % of monoclonal lesions progress and 64% polyclonal lesions regress(38).

Cellular origin of squamous intraepithelial lesions:

Three sites have been proposed as the site of origin of squamous intraepithelial lesions (SIL). They are basal cells of the squamous epithelium of the portio, basal cells of the transformation zone and reserve cells of endocervix(39). Transformation zone is the most common site for origin of SIL. About 10% of SIL can occur in the endocervical canal without squamocolumnar junction involvement(40). So SIL involving exocervical surface is considered as low grade and SIL extending into endocervical canal is high grade. From these observations, now it is proposed that most SIL arises in the basal cells of the transformation zone. In unscreened population, high grade cervical cancer precursors are more common than invasive cervical cancer. This suggests that only few

high grade precursors have the capacity to progress to invasive cervical cancers(41). Studies have shown that on long term follow up, partially treated or untreated HSIL had 30-50 % chance for progression to invasive cervical cancer over 30 years(42).

Clinical features:

SIL is seen more commonly on the posterior lip of cervix and it involves rarely the lateral cervical regions(43). It may spread horizontally and involve the entire transformation zone. Rarely endocervical extension beyond the endocervical canal and then into the uterus can be seen. Based on the severity of the lesion, the size and endocervical distribution tend to vary.

Screening tests:

Pap smear:

Even though pap smear is the best screening test and has reduced significant number of cervical cancer deaths, there are limitations. Sensitivity of one Pap smear to identify cervical intraepithelial lesions ranges from 30-87%(44). Approximately 30 % patients who are diagnosed with cervical cancer have at least one previously false negative Pap test.

Criteria for precancerous cervical lesion:

According to the Bethesda 2014, squamous cell abnormalities are classified as following:(45)

1) Atypical squamous cells
    - of undetermined significance (ASC-US)
    - cannot exclude HSIL (ASC-H)
2) Low-grade squamous intraepithelial lesion (LSIL)
3) High-grade squamous intraepithelial lesion (HSIL)
4) Squamous cell carcinoma.

Table 1: Criteria used to diagnose squamous cell abnormalities

Bethesda system	LSIL	HSIL	
CIN terminology	CIN 1	CIN 2	CIN 3
WHO terminology	Mild dysplasia	Moderate dysplasia	Severe dysplasia
Cell type	Superficial or intermediate	Parabasal	Basal
Cell arrangement	Singly or sheets	Singly or sheets	Singly or sheets
Number abnormal cells	+	++	+++
Koilocytosis	+++	+	+/–
Nuclear size	+++	++	+
Hyperchromasia	+	++	+++
Nuclear:cytoplasmic ratio	+	++	+++

Adapted from Blaustein's Pathology of the Female Genital Tract - Sixth Edition

Bethesda System subdivides the ASC category into two subdivisions.

- Atypical squamous cells of undetermined significance (ASCUS) refers to samples in which the cytological changes are suggestive of LSIL, but lack sufficient cytological abnormalities to allow a definitive diagnosis.
- Atypical squamous cells – cannot exclude HSIL (ASC-H) refers to samples in which the cytological changes are suggestive of HSIL but not insufficient to allow a definitive interpretation.

1. Atypical squamous cells of undetermined significance(ASC-US)

Criteria:

- In ASC-US cells nuclei are enlarged from 2.5to 3 times of the normal intermediate cell nucleus or more than 2 times the nucleus size of a squamous metaplastic cell.
- Mildly increased N/C ratio with minimal nuclear hyperchromasia and irregularly distributed chromatin.
- Atypical parakeratosis and incomplete koilocytosis.

2. Low grade squamous Intraepithelial lesion (LSIL)

Criteria:

- Nuclear enlargement > 3times of normal intermediate nuclei with mildly increased N/C ratio.
- Cytoplasmic and nuclear changes usually seen in intermediate or superficial squamous cells.
- Overall cell size is enlarged with abundant cytoplasm.
- Nuclei are usually hyperchromatic with coarsely granular to densely opaque chromatin, mild anisonucleosis and absent nucleoli.
- Smooth to irregular nuclear membranes.
- Cells with koilocytosis should also have nuclear abnormalities to be considered as LSIL.

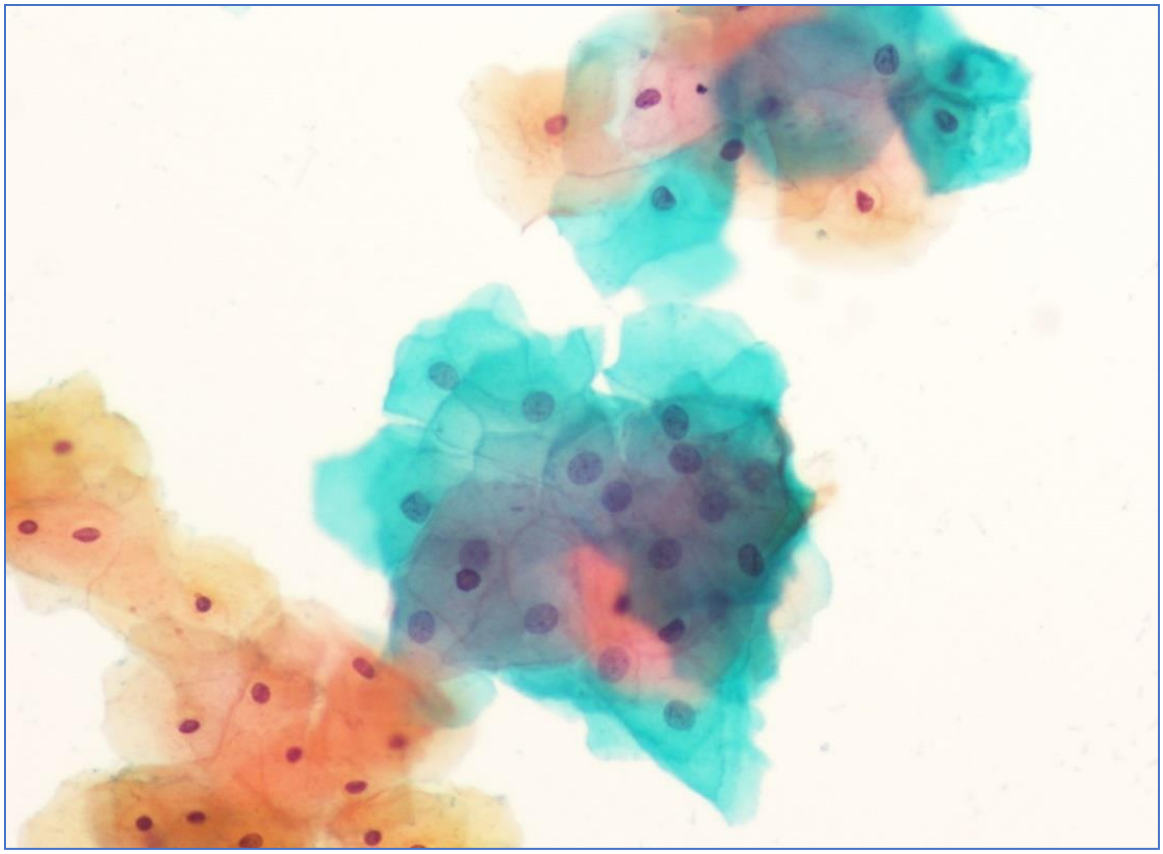


Figure 2: Atypical squamous cell of undetermined significance at 200x

ASC-US (LBP, thin prep) - Cohesive sheets of cells shows nuclear enlargement of more than two and half times of nucleus of intermediate cells.

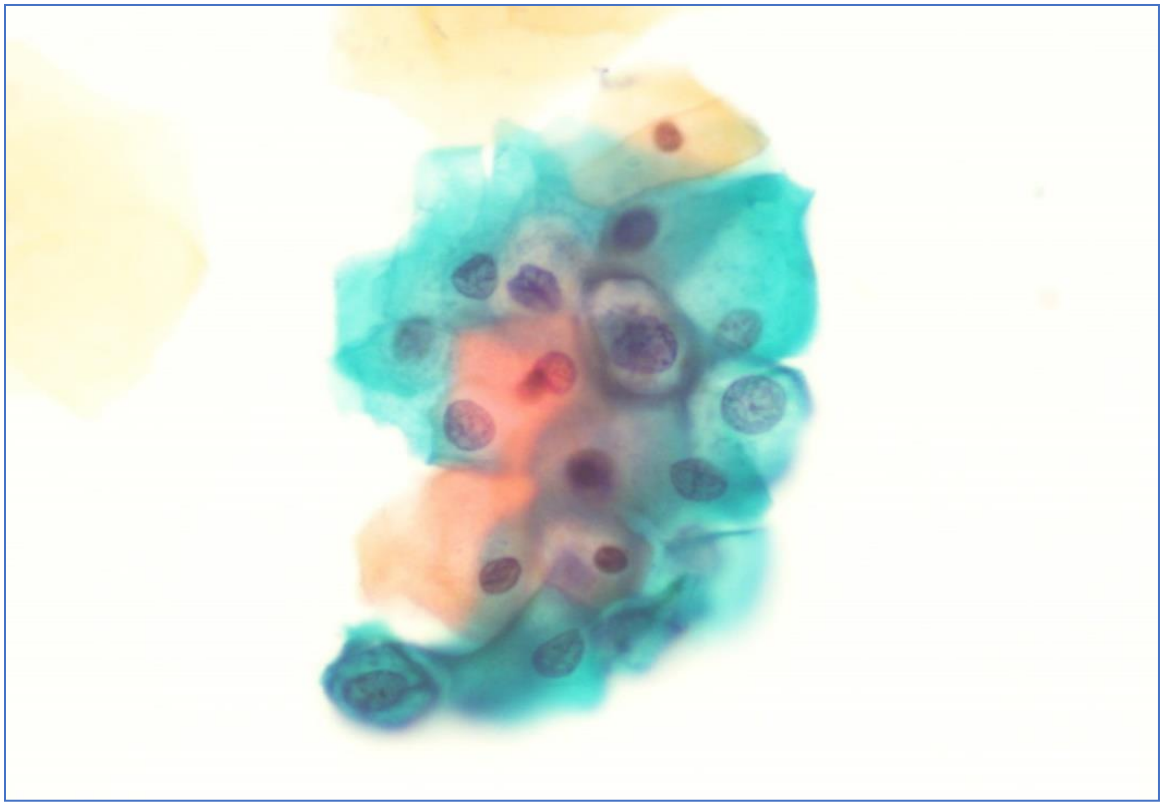


Fig 3: Low grade Squamous Intraepithelial Lesion(LSIL)at 400X

LSIL (LBP, thin prep) - Intermediate cells with koilocytic change with perinuclear halo, nuclear enlargement and few with nuclear membrane irregularity.

Atypical squamous cells – cannot exclude HSIL (ASC-H):

Criteria:

- Atypical squamous cells – cannot exclude HSIL (ASC-H) - cytological changes are suggestive of HSIL but not insufficient to allow a definitive interpretation.
- Cells are size of metaplastic cells usually seen singly or arranged in small groups.
- Nuclei about 1.5–2.5 times more than normal metaplastic squamous cells with high N/C ratio.

High-Grade Squamous Intraepithelial Lesion (HSIL):

Criteria:

- HSIL cells are smaller and show less cytoplasmic maturity as compared to cells of LSIL but higher N/C ratio.
- Cells occur singly, sheets, or in syncytial-like aggregates may result in hyperchromatic crowded groups.
- Nuclei are generally hyperchromatic with irregular nuclear grooves and frequent indentation.
- Nucleoli are usually not seen, but may be present when high grade lesion extends into endocervical gland.
- Cytoplasm is usually immature but occasional “mature” and densely keratinized cytoplasm may be seen.

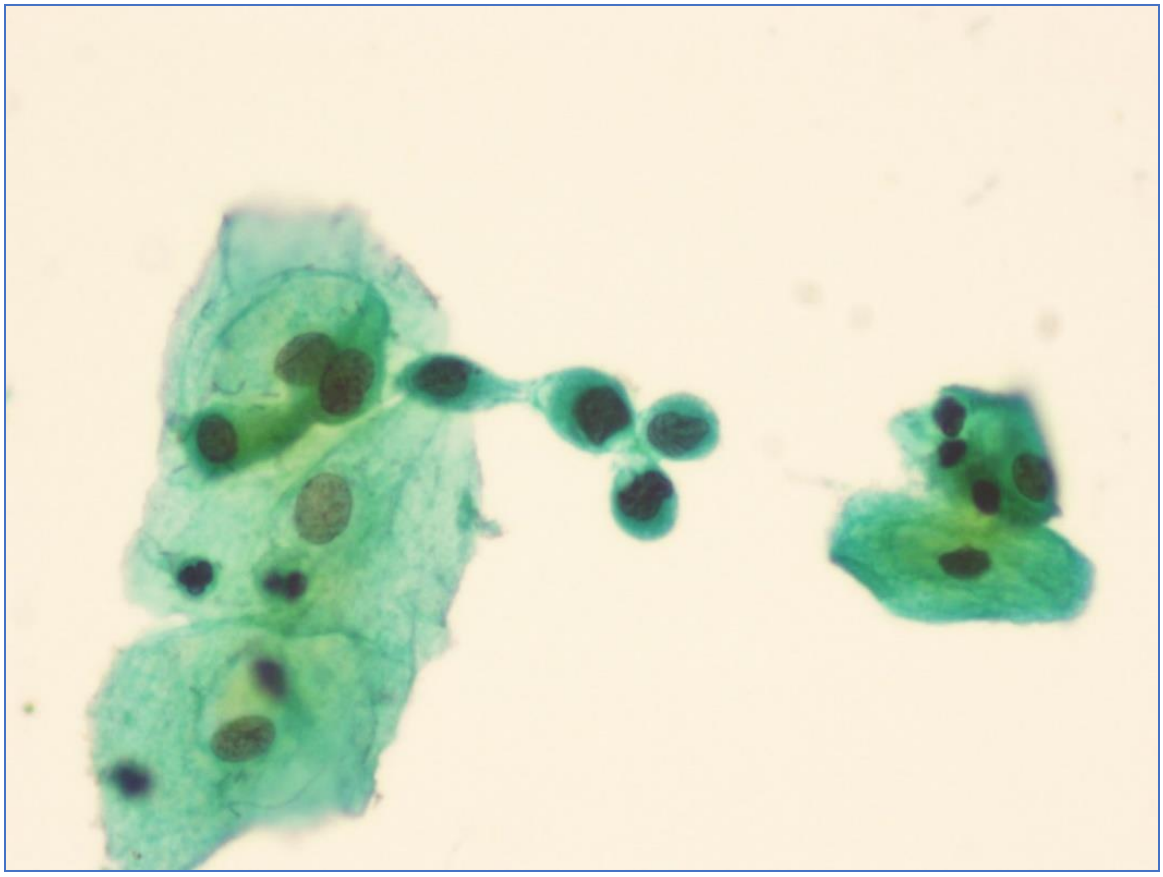


Figure 4: Atypical Squamous cell – Can not exclude HSIL (ASC-H) at 400x

ASC-H (LBP, thin prep) - A few metaplastic squamous cells with enlarged nuclei, high nuclear to cytoplasmic ratio, coarse chromatin and irregular nuclear contour.

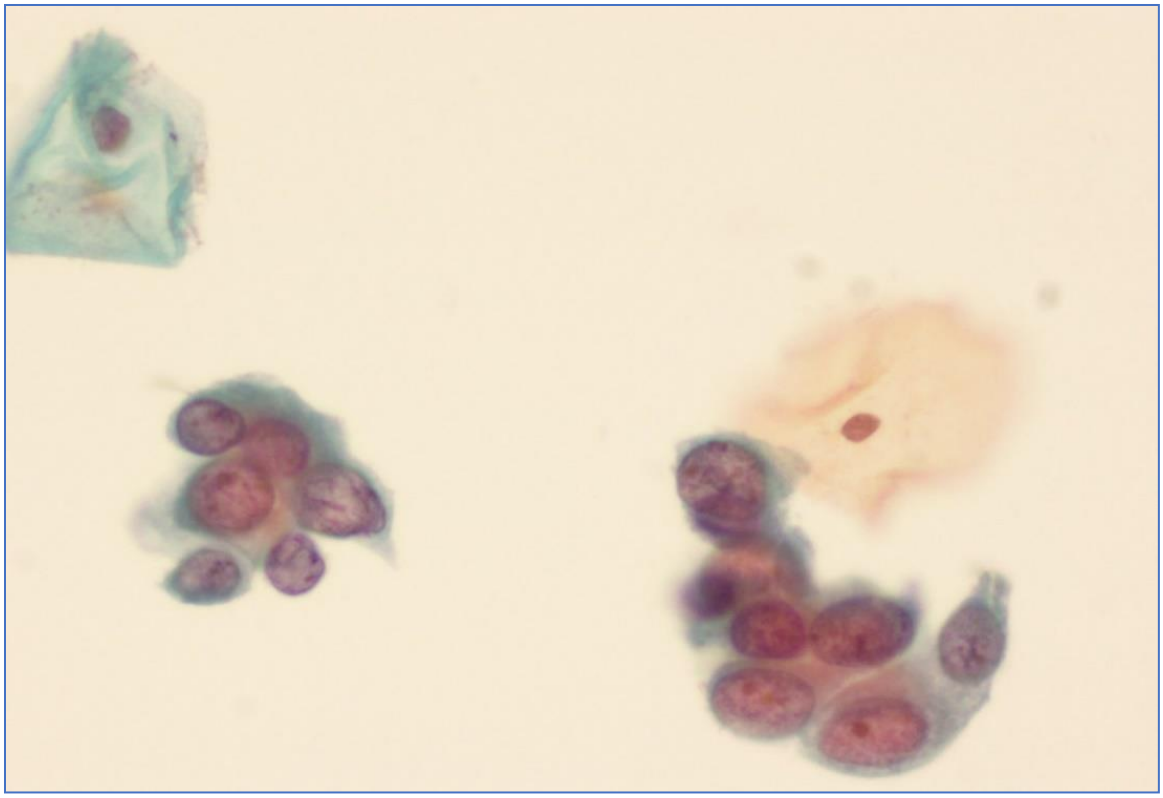


Figure 5: High grade squamous intraepithelial lesion (HSIL) at 400x

HSIL (LBP, thin prep) - Clusters of cells with high N/C ratios, fine granular chromatin, few with visible nucleoli and an occasional nuclear groove.

Management of precancerous cervical lesions:

The American Society for Colposcopy and Cervical Pathology (ASCCP)guidelines(18):

Atypical squamous Cells of Undetermined Significance: (ASC-US)

- Women with ASC-US who are HPV-negative should come for repeat HPV testing or co-testing at 3 years.

- Women with ASC-US who are HPV-positive should be referred for colposcopy examination to look for precancerous lesion.

- If hr-HPV test is not available, suggested follow-up at 12 months.

- If colposcopy is negative, co-testing is recommended at 12 months. If both tests are negative (Cytology as well as HPV), suggested follow up testing in 3 years.

- Diagnostic excisional tests like loop electrosurgical excision is not acceptable in women with ASC-US cytology with absence of high grade (CIN2+) cervical intraepithelial lesion.

- Follow-up of ASC-US in special populations: women aged 21–24 years, women aged 65 years and older, pregnant women, and postmenopausal women.

Low-Grade Squamous Intraepithelial Lesion (LSIL)

- ASCUS-LSIL Triage Study (ALTS) suggested that high-risk -HPV testing is not recommended particularly in women with less than thirty years due to the high prevalence of HPV infection(46).

- HPV testing is advisable for LSIL in postmenopausal women because of more specificity in this group of women.

- HPV co-testing in women with LSIL cytology is recommended above 30 years of age.

- Follow-up is recommended in women with LSIL cytology less than 25 years of age at the interval period of 12 months

- In women above 25 years and older, co testing is recommended in 3 years, if HPV test result is negative, whereas colposcopic examination is suggested if HPV results is positive.

Atypical Squamous cells – Cannot exclude HSIL(ASC-H)

- Colposcopy guided cervical examination is recommended for women with ASC-H cytology irrespective of HPV result. Reflex HPV testing is not advisable in ASC-H cytology.

High-Grade Squamous Intraepithelial Lesion (HSIL)

- Colposcopy guided cervical examination is recommended for women with HSIL cytology. HPV testing is not recommended.

- Most of the women with a cytologic result of HSIL will have identifiable lesion in colposcopy and biopsy of lesion should be taken to confirm high grade (CIN 2+) cervical intraepithelial lesion(47).

- Women aged 25 years and older with cytologic HSIL, immediate excisional procedure may be performed at the time of colposcopy if a lesion is identified.

- If biopsy does not show high grade intraepithelial lesion (CIN 2+), review of cytology as well as histology slides are required. For difficult cases p16 immunohistochemistry, may be helpful in identification of the lesion(48).

Treatment:

Colposcopy with biopsy helps in planning the management of patients with precursor lesions of cervix. It allows the gynaecologist to rule out invasive cancer. These precancerous cervical lesions will be treated by conservative methods like laser ablation, cryosurgery and loop electrical excision procedure (LEEP) or cone biopsy.

Prognosis:

High grade precursor lesions are more likely to persist. Approximately 57% of CIN 1 lesions regress spontaneously without any intervention and 11% progress to carcinoma in situ. About 43% of CIN 2 lesions regress while 22% progress to carcinoma in situ. 32% of CIN III lesions regress and 12 % progress to carcinoma in situ(49). Recent studies by Castle et al. shows that rates of spontaneous regression of biopsy confirmed CIN II after 24 months of follow-up was 43% which is identical to older studies(50).

HPV DNA testing.

Because of known association between high risk HPV infection and cervical cancer, hr-HPV DNA testing becomes a very important test in forming strategies for cervical cancer screening. Initially it was approved as a triage test which improved the detection of high grade cervical lesion in smears with minor cytological abnormalities compared with repeat cytology. Though, hr-HPV DNA test is useful to triage smears with minor cytological abnormalities, it has low specificity due to inability to differentiate between transformed HPV infection and transient HPV infection. The hr-HPV DNA testing can be done on liquid based cytological specimens.

Since the knowledge of HPV pathophysiology has been established, the identification of new biomarkers with ability to distinguish those at risk of disease progression becomes necessary. Therefore several host cell biomarkers were evaluated to improve the specificity for the screening of cervical intraepithelial lesions(3). One such bio marker which has been recently identified is dual P16INK4A/ki67.

p16 INK4A:

P16 INK4A (also referred to as p16) is a tumor suppressor protein that helps in cell cycle regulation. It acts by inhibiting cyclin D – cyclin dependent kinase 4 complex formation, thereby preventing cell cycle progression. Numerous studies have demonstrated p16 to be down regulated in many tumors. However, interestingly, increased expression of p16 has also been described in few tumors (51).

Physiological role of p16Ink4a:

P16 belongs to the Ink4 family of cyclin dependent kinase inhibitors. The CDKN2A gene encoding this protein is located on chromosome 9p21 within the INK4a/ARF locus. This gene codes for two different proteins which inhibit cell cycle progression - p16Ink4a and p19ARF.

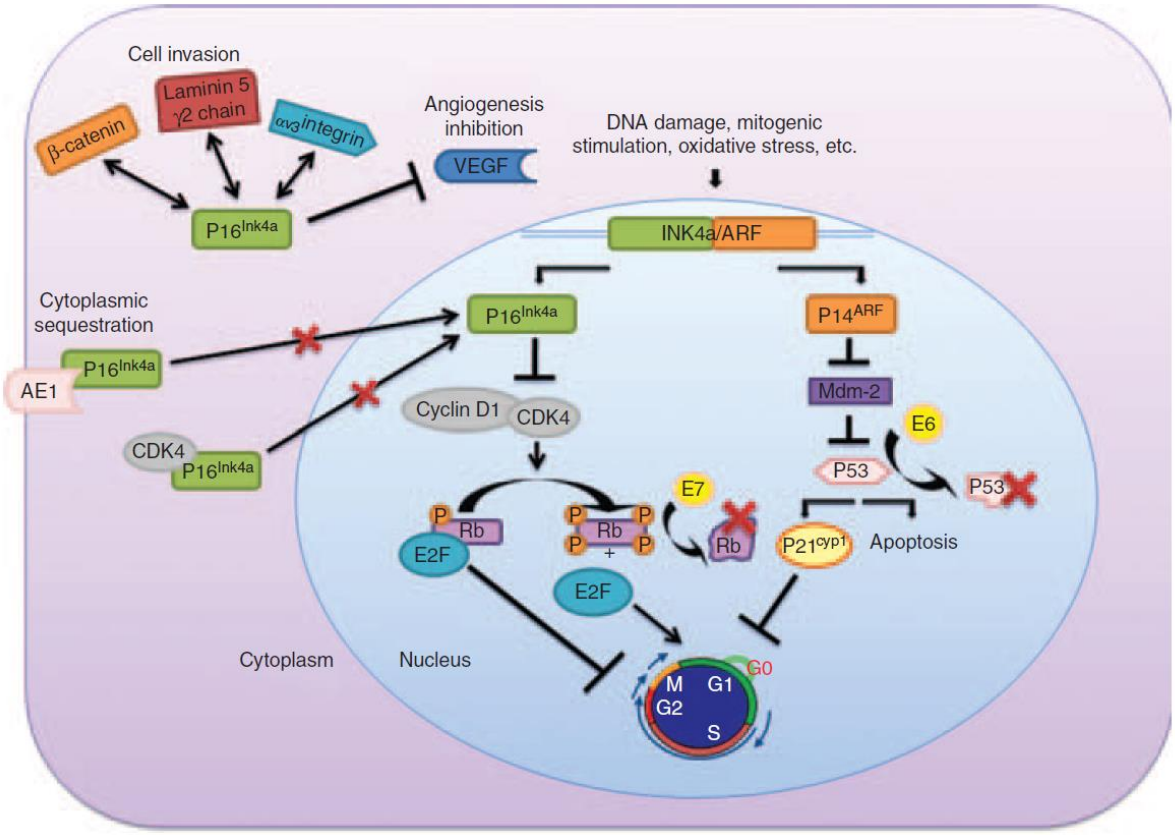


Fig 6: Molecular mechanism of cervical cancer by HPV

Adapted from Romagosa et al, Oncogene (2011)

P16Ink4a is involved in the Retinoblastoma pathway (Rb), whereas p19ARF acts via the p53 pathway. These proteins act as tumor suppressor proteins by complex interactions. (52,53). Retinoblastoma (RB) protein is a crucial negative regulator of cell cycle progression from the G1 phase to S phase. Within quiescent cells, RB protein in its active hypo phosphorylated state complexes with E2F transcription factors that are required for the expression of genes which help in progression to S phase. This prevents G1-S transition and causes cell cycle arrest. However, when RB protein is phosphorylated by

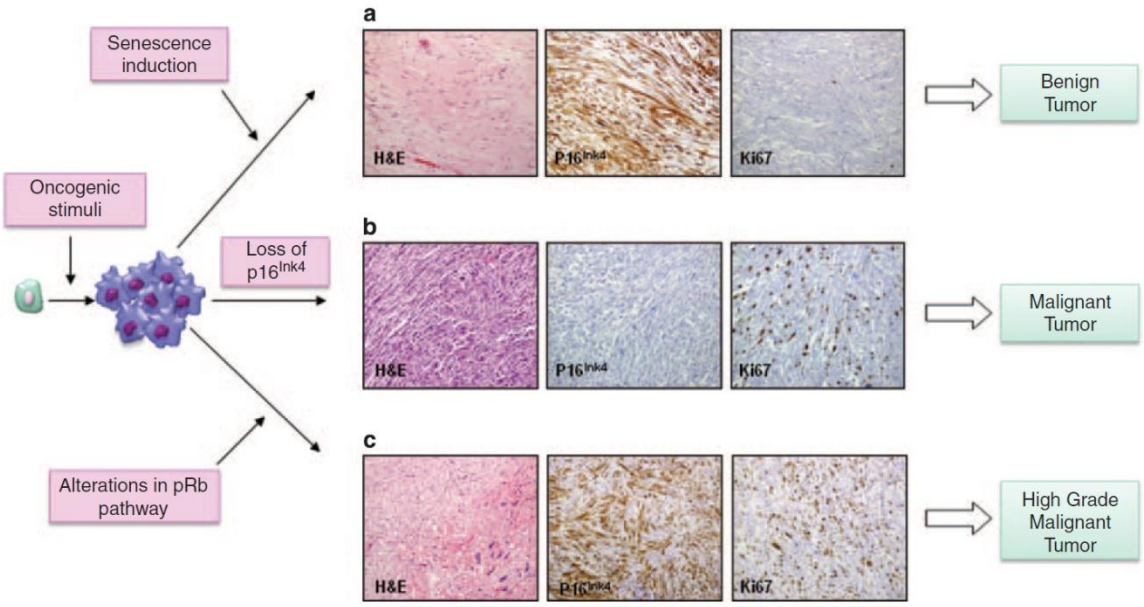
cyclin D – cyclin dependent kinase 4/6 complex, it releases the E2F transcription factors, and thereby facilitates cell cycle progression.(53). P16Ink4a acts as a CDK inhibitor, blocks the cyclin D – CDK4/6 mediated phosphorylation of Rb and induces cell cycle arrest. In HPV-related neoplasms, the molecular mechanism if p16 INK4a can be explained by the presence of HPV oncoproteins E6 and E7. The integration of the viral DNA into host DNA causes overexpression of these viral oncoproteins. E6 protein binds and causes degradation of p53. E7 binds to RB protein and displaces the E2F transcription factors promoting cell cycle progression(54,55). This inactivation of the RB protein releases p16 INK4a from its negative feedback control, which in turn causes a paradoxical increase in the levels of p16. Therefore, the overexpression of p16 INK4a in HPV related tumors is considered to be due to an unsuccessful attempt to inhibit cell replication(56). In addition to its role in cell cycle regulation, p16 INK4a has also been implicated in few other cellular processes including apoptosis, cell invasion and angiogenesis and it has been hypothesized that this role may contribute to its overexpression in some neoplasms(57).

Subcellular location of p16Ink4a overexpression:

Cell cycle regulation has been the classic function related to p16 INK4a and this characteristically take place in the nucleus. However, there is considerable recent evidence of significantly increased levels of p16 INK4a in several neoplasms and this has also been shown to be associated with tumor progression in few of them(58). It has been

hypothesized that p16 INK4a has varied roles in varied subcellular locations, and that the nuclear p16 INK4a mainly regulates cell cycle. This has been supported by studies that show downregulation of nuclear p16 INK4a was associated with E2F overexpression. However, overexpression of cytoplasmic p16 INK4a exhibited no relation with E2F expression(59). Many theories have been postulated to explain the presence of cytoplasmic p16 INK4a, and it seems this is unrelated to p16 INK4a gene alteration. Proteomic and post translational studies have shown that p16 INK4a is expressed in different subcellular location, depending on post-translational modification or its ability to form complexes with other proteins(60). In quiescent cells, p16 INK4a forms a complex with CDK4 and prevents its subsequent kinase function.

The p16 INK4a/CDK4 complex is a large complex that is unable to pass through the nuclear membrane. However, in neoplastic cells, the localization of p16 INK4a in the cytoplasm due to CDK4 sequestration is considered to be an indirect effect of the RB pathway alteration. Anion exchanger protein 1 (AE1) is another transmembrane protein that causes sequestration of p16 INK4a in the cytoplasm with co-accumulation of both proteins. Also, p16 INK4a interacts with numerous cytoplasmic proteins, such as α-β-γ actin, α-β tubulin and CDK4/6(61). It is important to note that further work is still needed to elucidate the molecular mechanisms of p16 INK4a cytoplasmic localization and its connection with failure of the tumor suppressor function of p16 INK4a.



Adapted from Romagosa et al, Oncogene (2011)

Figure 7: Molecular difference between expression of p16 and Ki-67 among different tumors.

The role of p16 in different tumors is explained in the above picture. The first picture shows schwannoma with overexpression of p16, which is linked to the senescence induction due to the oncogenic stimuli, associated with low ki67 index. The second picture shows malignant peripheral nerve sheath tumor, with loss of p16 expression and associated with high ki67 proliferative index. The third picture shows an undifferentiated sarcoma with overexpression of p16 as well as ki67 index, due to alteration in the Rb pathway(51).

Ki-67:

Cell proliferation is strictly associated with high expression of ki-67 protein in human beings. This ki-67 antigen is exclusively identified within the nucleus during interphase but in mitosis majority of this antigen is seen over the surface of chromosomes. Hence in all active phases of cell cycle ki-67 protein is expressed (G1, S, G2 & mitosis). Ki67 protein is absent during resting phase(G0). So, it is used to calculate the growth fraction of cells. Even though this ki-67 protein is well identified in molecular levels as good indicator of proliferation, functional characteristics still remains unclear. Ki-67 protein expression is a must for progression through the cell-division cycle(62).

P16/ki-67 dual immunocytochemistry:

p16 immunohistochemistry is used for identification of p16 overexpression in cervical biopsies which indicates oncogenic transformation caused by persistent hr-HV infections. However, in 2011 CINtec PLUS immunocytochemistry assay was introduced in cytology which provides simultaneous qualitative detection of p16 and Ki-67. P16 is the cyclin-dependent kinase inhibitor. From the molecular pathogenesis, increased expression of p16 with activation of E2F is identified as a response to incorporation of HPV E7 oncoprotein with RB gene. Ki-67 is used as proliferative index for assessment of growth fraction of cell cycle. Combining p16 with ki-67, detects the HPV infected cells undergoing transformed and uncontrolled proliferation.

There are several studies done on the performance of dual immunocytochemistry for p16 and Ki-67 for the identification of CIN2+ lesion(63–67). Bergeron et al assessed performance of P16/Ki-67 dual immunocytochemistry in a group of 28,000 women, from 5 European countries. In that study, performance of hr-HPV test and p16/Ki-67 dual immunocytochemistry were compared among women with the diagnosis of ASCUS and LSIL. Sensitivity of p16/Ki-67 in ASCUS for the detection of CIN II+ was 92.2% (90.9% for HR HPV test), specificity was 80.6% (36.3% for HPV test). In a group of LSIL, sensitivity was 94.2% (96.4% for hr-HPV test), specificity was 68.0% (19.1% for hr- HPV).

Petry et al performed the utility of p16/Ki-67 dual immunocytochemistry for the detection of CIN II+ lesions in women with hr-HPV positive tests. p16/Ki67 showed sensitivity of 91.9% for CIN II+ and specificity was 82.1% for CIN II. Wentzensen et al compared the results of p16/Ki67 immunocytochemistry with high risk HPV test in identification of CIN II+ lesions in colposcopy referral population. Sensitivity was 85.5% and specificity 59.4% for CIN II whereas for CIN III sensitivity was 90.6% and specificity was 48.6%. These studies showed that specificity of dual immunocytochemistry is higher as compared to hr-HPV test, in detection of underlying high grade cervical intraepithelial lesion. Hence, p16/ki-67 can be used as a triage tool for minor cytological abnormalities i.e. low grade squamous intraepithelial lesion(LSIL) and atypical cells of undetermined significance (ASC-US).

In all the above studies co-expression of cytoplasmic p16 and nuclear Ki-67 in at least one cell was considered as positive ICC test which indicates transformed infection. The concept of single positive cell as a cutoff has been used due to the monoclonal nature of any malignant neoplasm. But these studies also showed that false positivity for p16/Ki-67 resulted in decrease in its positive predictive value to identify underlying high-grade lesion.

According to recent literature by Peter Zeimke, when a higher cut off of 10 cells was used, the probability of underlying high-grade lesions (CIN2+) also increased in a sample. Using a score of 10 cells as a positive result instead of 1 cell led to significantly improved specificity (89 vs 70.2%) and positive predictive value(55.7%vs 46.7%) among women in LSIL group(68). Interpretation of p16/Ki-67 dual immunocytochemistry is morphology independent: thus, limited training is needed for interpretation and also this test has good reproducibility. A few studies have evaluated the performance of p16/Ki-67 in urine cytology also. P16 over expression in urothelial carcinoma is independent of high-risk HPV oncoprotein. In urothelial carcinoma p16 expression is most often undetectable. It is due to either deletion or mutation of CDKN2A. In contrast, over expression of p16 has been reported in urothelial lesions up to 80% of high grade tumors and 100% of carcinoma in situ cases. It may be due to mutation of E2F transcription factor gene or alteration of Rb gene, resulted in phosphorylation of the Rb protein and uncontrolled progression of the cell cycle. p16 overexpression indicates aggressive disease and may be used for prognostic and therapeutic purpose.

Clinical utility of p16/ki-67 in urothelial malignancies has also been assessed in a few studies. These studies propose that dual immunocytochemistry is used in evaluation of high grade urothelial cancer and for the follow-up management after conservative treatment for non- invasive urothelial carcinoma (69,70).

The purpose of this study is to assess the significance of p16/ki67immunocytochemistry in improving the predictive value for high grade cervical intraepithelial CIN2+lesions.

MATERIALS AND METHODOLOGY

After approval from the Institutional Review Board (IRB) min no: 10175, this prospective diagnostic study was conducted in the Department of Pathology, in conjunction with Department of Virology and Department of Gynaecologic oncology.

Study population:

A total of 175 patients from gynecology/ gynecologic oncology, whose cervical smears were reported by the Department of general pathology as ASC-US/LSIL/ASC-H and HSIL, were identified for the study, over a period of 8 months from August 2016 to March 2017. Information sheet and consent form were given to all patients willing to participate in this study.

Exclusion criteria:

All patients, who were

- Less than 25 years,
- Pregnant women,
- Patients unwilling for colposcopy/ biopsy,
- Seropositive patients and
- Patients previously treated for CIN were excluded from this study.

Study design

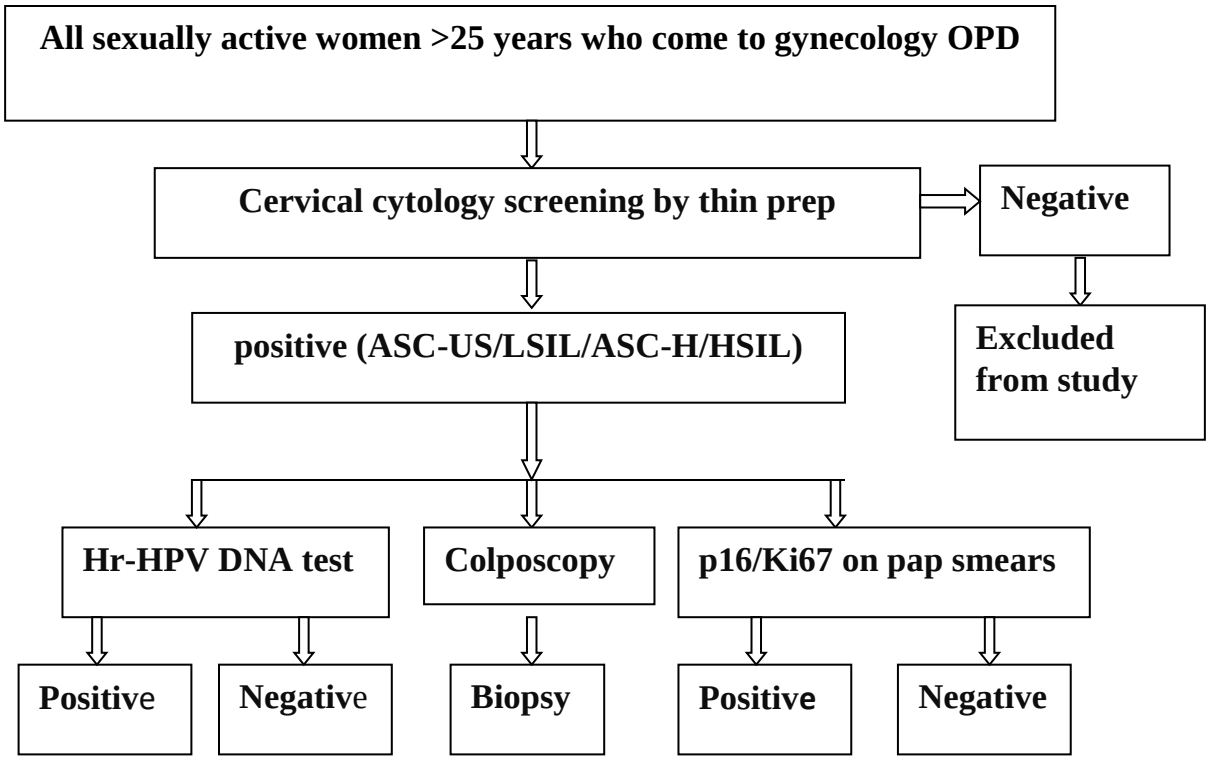


Figure 08: Detailed diagrammatic Algorithm of the study.

Sample collection and processing:

Thin prep cervical smear:

The residual Thin prep sample material of all study patients (Hologic vials) were stored at 2-4 degree Celsius for up to 6 weeks. If the samples exceeded 6 weeks of storage period, they were processed in the Thin Prep® 2000 Processor (Hologic™ Inc.) and the unstained smears were stored in ethanol fixative at 2-4degree Celsius.

The dual immunocytochemistry was performed in batches. In view of inadequacy and poor cellularity of some smears that were stored, the third batch of smears were prepared from the refrigerated samples, on the day of the ICC procedure.

Technical procedure as described in Appendix-1.

P16/ki67 dual immunocytochemistry:

Immunocytochemistry analysis using the CINtec PLUS Cytology kit (Roche MTM Laboratories, Heidelberg, Germany) were done on these smears according to the manufacturer's instructions. All slides were examined by the principal investigator and the guide.

Technical procedure as described in Appendix-2.

Hr-HPV DNA test:

HPV DNA tests for hr-HPV were also done on all study patients as per the standard management protocol followed in the Gynecology outpatient department. The material for this test was collected at the time of colposcopy in using the digene HC2 DNA collection device which consisted of a cervical brush and digene Specimen Transport Medium(STM). In vitro nucleic acid assay with signal amplification by Hybrid Capture 2 assay (HC2; Digene Corp., Gaithersburg, MD) was used.

Technical procedure as described in Appendix-3.

Interpretation of HPV DNA:

The hr - HPV DNA data was interpreted based on the results (positive/ negative) obtained from Hybrid Capture (HC2) in the department of virology. Serial amplification assay for hr- HPV DNA in cervical specimen can detect upto13 different hr-HPV DNA types [16,18,31,33,35,39, 45,51,52,56,58,59 and 68] in the cervical cells. Detection of hr-HPV DNA in Hybrid Capture (HC2) technique is done by using microplate chemiluminescence. The result is obtained as relative light units (RLU). The digene analysis hr-HPV DNA test cut off of 1 pg/ml is equivalent to 1,00,0000 HPV copies /ml or 5,000 copies per assay. STM (Specimen Transport Medium) specimen with RLU/cutoff Value ratios equal or greater than 1 are considered ‘positive’. Specimens with RLU/cutoff value ratios<1 considered ‘Negative’ or ‘None detected’ for the 13 HPV types tested.

Colposcopy and biopsy:

Colposcopy and biopsy were also done on all the study patients as part of a routine protocol, in the Gynecology outpatient department, according to accepted diagnostic standards.

Histopathology:

Fixed tissue samples were processed to paraffin blocks in the histopathological laboratory. Slides(4μm) were cut on the microtome, stained with hematoxylin and eosin (H+E) and were assessed by histopathologists who were blinded to the ICC and HPV results of the study patients. Biopsy was considered as gold standard, against which the new dual immunocytochemistry and hr-HPV DNA results were correlated.

Interpretation of P16/ki-67 dual immunocytochemistry:

The dual immunocytochemistry was interpreted by the primary investigator and guide. The presence of one or more cervical epithelial cells with co-localization of brown cytoplasmic immunostaining and red nuclear immuno-staining within the same cell was regarded as a positive CINtec PLUS test result. We also assessed the performance of p16/Ki-67 dual immunocytochemistry, with a cut off for positivity as more than ten cells.

If there is no cervical epithelial cell showing simultaneous brown cytoplasmic immuno staining and red nuclear immuno-staining, the CINtec PLUS test result is considered negative.

The presence of cervical epithelial cells that do show a single immuno-reactivity only for one of the two markers (e.g. – Brown staining for p16 only, or red staining for Ki-67 only)

is not considered as a positive test result for the CINtec PLUS kit. Strict criteria for positive and negative tests were followed to avoid discrepancy.

Smears from a known case of squamous cell carcinoma were used as a positive control, with colocalization of both brown cytoplasmic immunostaining (p16) and red nuclear immunostaining (ki67). Different cell types present in representative cervical cytology specimens, that are known to be negative for the expression of the p16 and ki-67 antigens (such as superficial cells) may serve as an additional internal negative control to assess any background staining.

Clinical details of the cases:

The clinical details of these patients were obtained from the charts retrieved from the Medical Records Department. The clinical features that were analyzed include age, indication for cervical screening, marital status, parity and menopause status.

Statistical method used

Sample size calculation:

The required samples size to show sensitivity of about 90% with 10% precision and 95% confidence interval was found to be 35 patients.

Clinical Research form:

The data obtained from each test and the necessary clinical information obtained from the clinical workstation were entered into the clinical research form for each case. (Form attached in annexure).

Data entry and analysis:

Data entry and analysis was done with Epidata software. Statistical analysis was performed by SPSS ststistics16.0 (statistical Package for Social Sciences, Version 16.0). Diagnostic statistics were used to assess the performance of the hr- HPV DNA test and p16/ki-67 dual immunocytochemistry. The sensitivity, specificity, positive and negative predictive values were calculated with corresponding 95% confidence intervals. To assess the statistical significance, we used P value as well as 95% CI difference in sensitivity, specificity, positive predictive value and negative predictive value between the tests. P value <0.05 was considered as statistically significant. Similarly, if 95% CI difference range does not include zero, it is considered statistical as well as clinically significant and if it has zero it was considered statistically not significant. Cohen's kappa agreement coefficient was used to compare the above two tests. Kappa values between 0.40 and 0.60 were considered to be moderate and those between 0.60 and 0.80 as good.

RESULTS

Study flow chart

Total number of study samples: **175**

Lost to follow up =**32**

Biopsy not done = **11**

HPV test not done = **08**

Total samples available for testing: **124**

Adequate	**Scanty**	**Inadequate(excluded)**
76	18	30

Total study cases: 94

Figure 9: Selection of cases included under study

A total of 175 cases were reported as ASCUS, LSIL, ASC-H and HSIL between August 2016 to March 2017. Thirty-two patients did not come for follow -up, eleven cases did not have biopsy and eight cases did not have HPV. Fifty-six cases were excluded from the study, leaving a total of 124 samples for further testing.

All slides were kept in 100% ethyl alcohol fixatives and stored in 2-3 degree Celsius. All tests were done in three batches in different time periods. Among 124 samples only 76 cases had adequate cellularity, 18 cases were paucicellular. Thirty cases did not have enough material to process, hence were excluded from the study, leaving a total of 94 women for inclusion in the study.

ABNORMAL PAP SMEAR GROUPS:

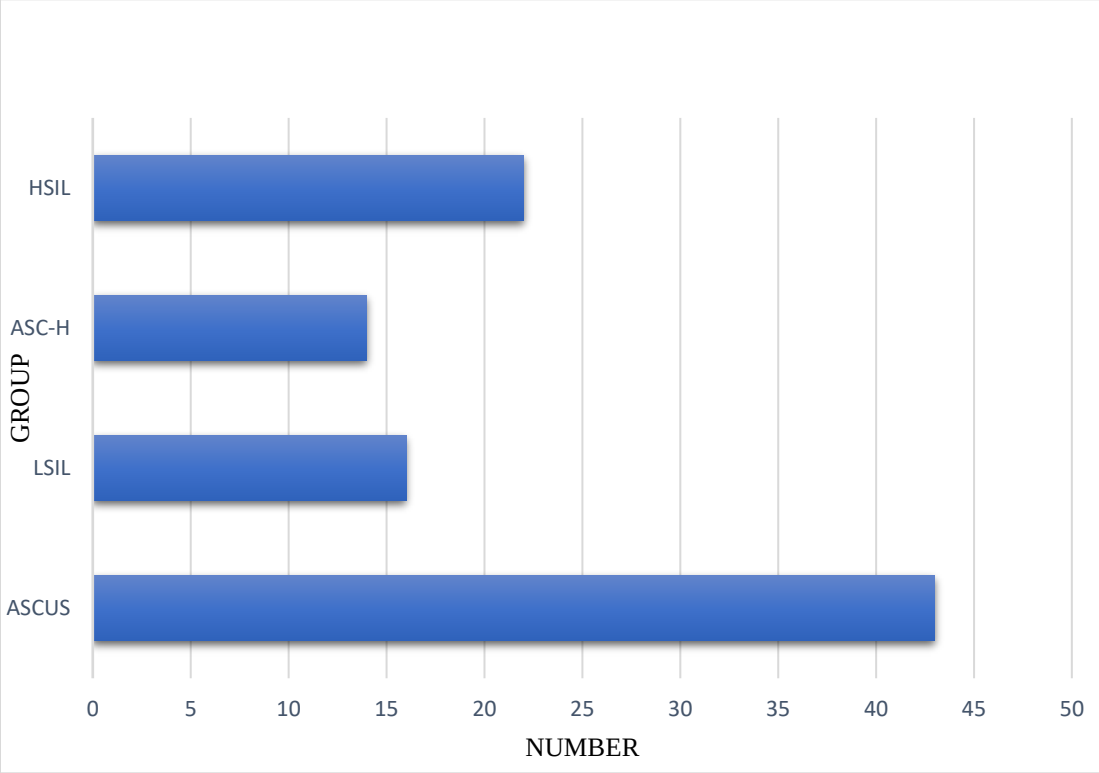


Figure 10: Number of cases in each abnormal pap smear groups

The median age was 42.73 years (standard deviation of 11.4 years) for overall abnormal pap smears with ASC-US, LSIL, ASC-H and HSIL. The youngest woman was 24 and the oldest patient was 71.

- ASC-US: 43 (45%)
- LSIL: 16 (17%)
- ASC-H: 14 (15%)
- HSIL: 22 (23%)

AGE:

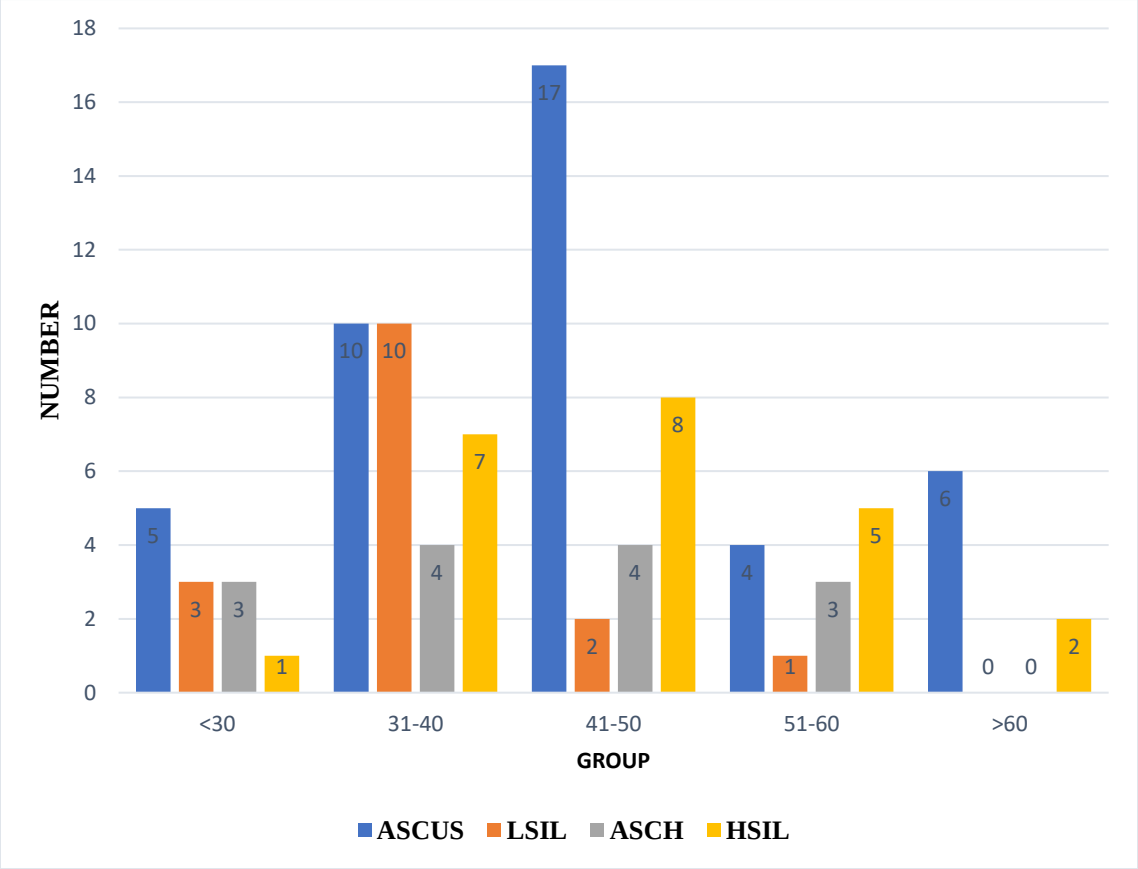


Figure 11: Different groups in accordance with age

ASC-US was the most common intra epithelial lesion identified in all age group of women.

There were 12cases (13%) less than 30 years of age and 82 cases (87%) above 30 years of age.

SCREENING:

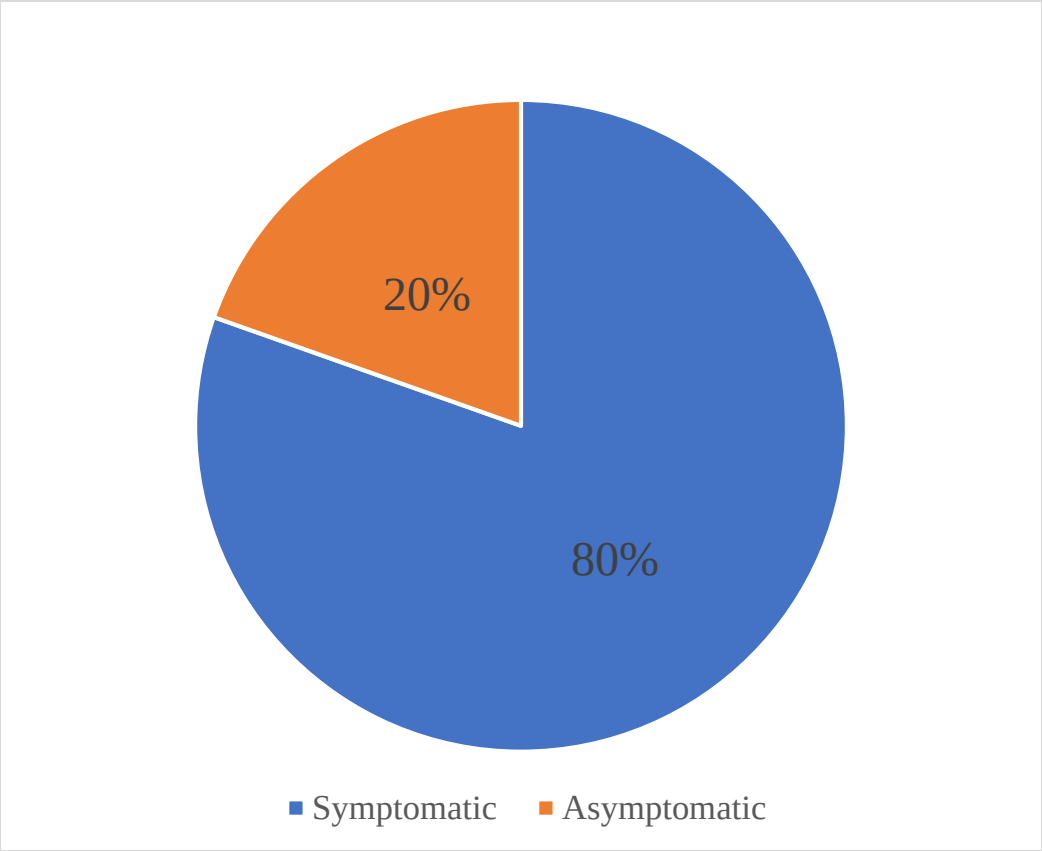


Figure 12: Indication for screening

Among 94 women, 75 were symptomatic (80%) whereas 19 were asymptomatic (20%) who were referred for routine screening.

SYMPTOMS:

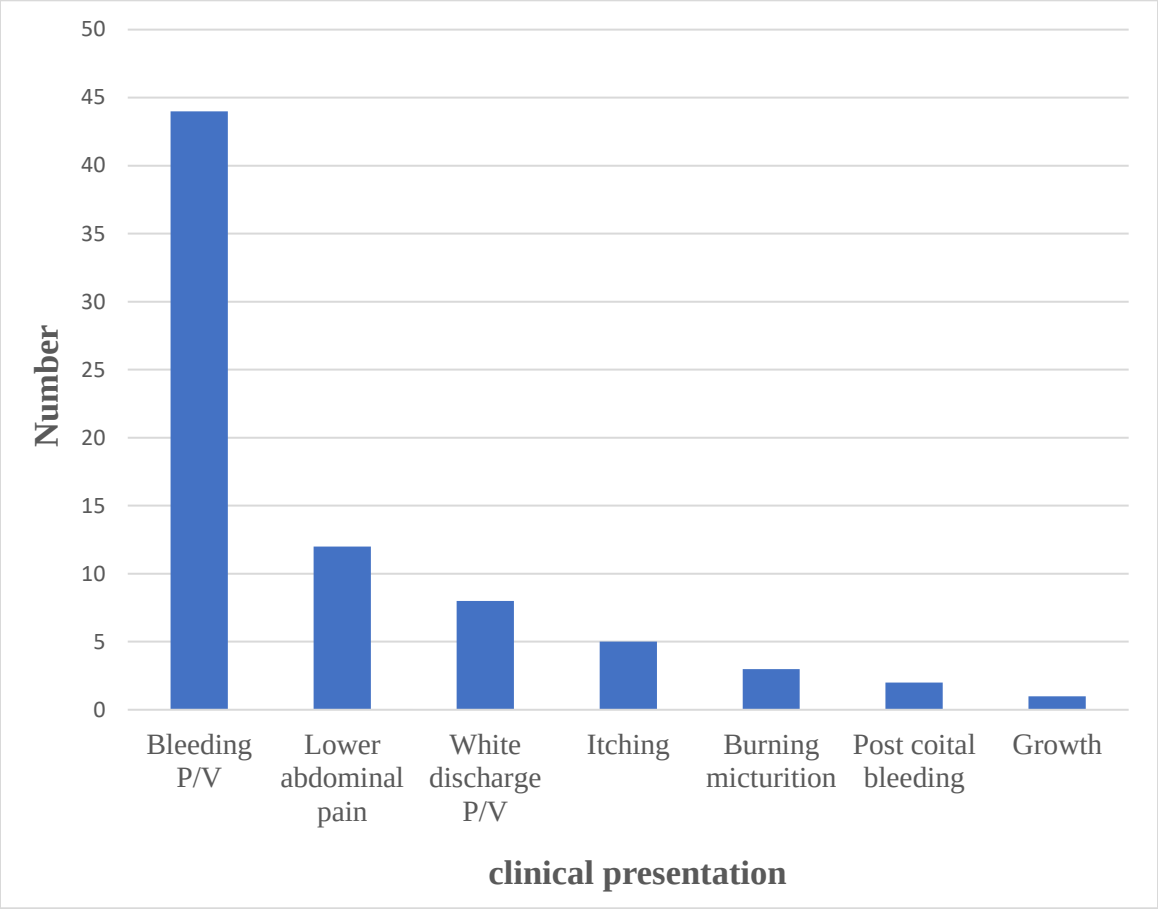


Figure 13: Different clinical presentation of abnormal pap smears

Symptomatic women presented with following complaints:

Bleeding per vagina (59%), lower abdominal pain (16%), white discharge per vagina (11%), itching (7%), burning micturition (4%), post coital bleeding (3%) and cervical growth (1%). Bleeding per vagina was the most common clinical presentation of women presented to gynecology/ gynecologic oncology.

MENSTRUAL STATUS:

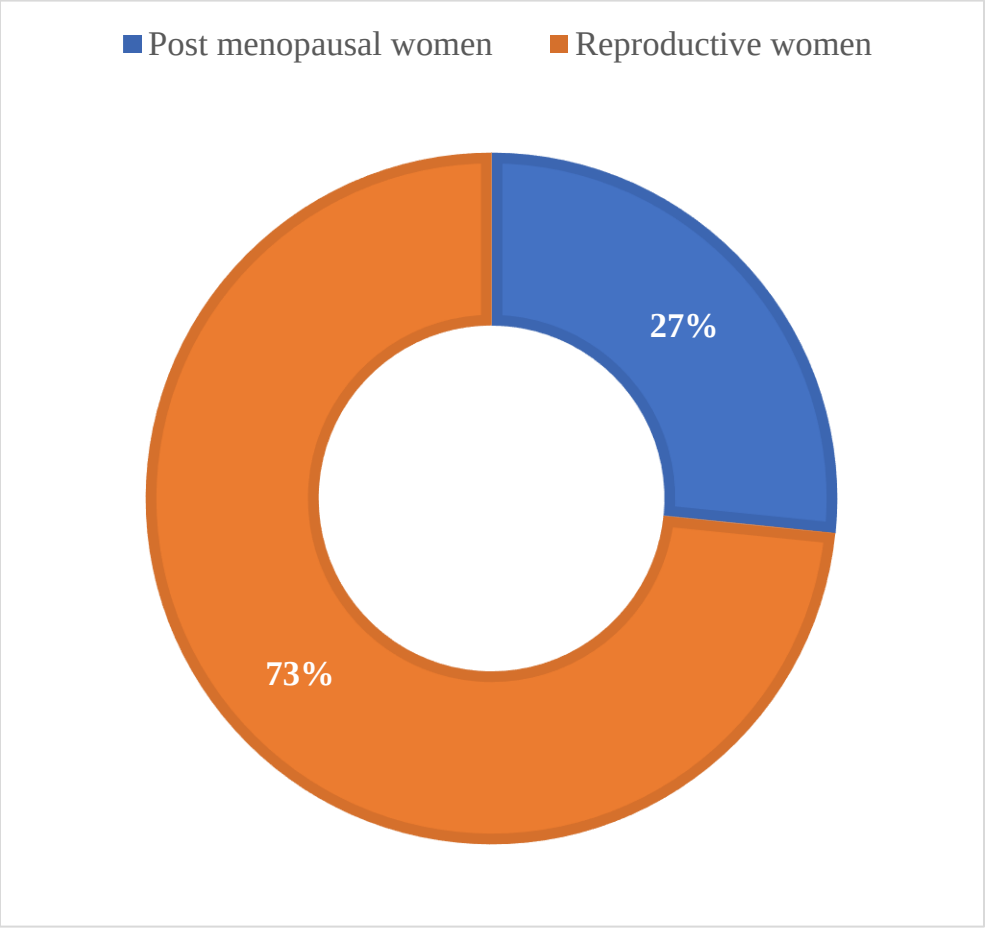


Fig 14: Number of reproductive and postmenopausal women

Among 94 women, 25 were in postmenopausal age group (27%) and 69 were reproductive age group (73%).

Hr-HPV TEST vs BIOPSY IN ≥ CIN2 LESIONS:

		≥ CIN2 LESIONS		TOTAL
		Positive	Negative	
hr-HPV test	Positive	30	28	58
	Negative	1	35	36
TOTAL		31	63	94

Table 02: hr– HPV test vs biopsy in ≥ CIN 2 lesions cross tabulation:

The above table shows performance of hr-HPV test among abnormal Pap smears as compared with the gold standard of ≥ CIN2 lesions i.e. biopsy. There were 94 study cases. Hr-HPV test was positive in 58 cases and negative in 36 cases. Among positive cases 30 cases (32%) had ≥ CIN2 lesions (True positive) and 28cases (30%) biopsies were negative (False positive). Among hr-HPV test negative cases, 1 case (1%) had ≥ CIN2 lesion (False negative) and 35 cases (37%) were negative (True negative) on biopsy.

- Total number of cases: 94
- Number of true positive: 30 (32%)
- Number of false positive:28 (30%)
- Number of false negative: 01 (1%)
- Number of true negative: 35 (37%)

P16/Ki-67 IMMUNOCYTOCHEMISTRY vs BIOPSY IN ≥ CIN2 LESIONS:

		≥ CIN2 LESIONS		TOTAL
		Positive	Negative	
p16/Ki-67	Positive	30	19	49
	Negative	1	44	45
TOTAL		31	63	94

Table 03: p16/Ki-67 vs biopsy in ≥ CIN2 lesions cross tabulation

The above table shows the performance of dual immunocytochemistry test among abnormal Pap smears as compared with gold standard of ≥ CIN2 lesions i.e. biopsy. There were 94 study cases. Dual immunocytochemistry was positive in 49 cases and negative in 45 cases. Among positive cases 30 cases (32%) had ≥ CIN2 lesion (True positive) and 19 cases (20%) biopsies were negative (False positive). Among negative cases 1case (1%) had ≥ CIN 2 lesions (False negative) and 44 cases (47%) biopsies were negative (True negative).

- Total number of cases: 94
- Number of true positive: 30 (32%)
- Number of false positive: 19 (20%)
- Number of false negative: 1(1%)
- Number of true negative:44 (47%)

PERFORMANCE OF P16/KI-67 AND HR-HPV TEST IN DETECTION OF ≥ CIN2 LESIONS:

	Sensitivity %(95%CI)	**Specificity %(95%CI)**	**PPV %(95%CI)**	**NPV %(95%CI)**
Hr-HPV test	96.8	55.8	51.7	97.6
P16/Ki-67≥ 1 positive cell	96.8	70.2	61.	97.6
P value	1.00	0.095	0.324	1.00
95% CI Difference	-09 to 09	-02 to 31	-09 to 28	-06 to 06

Table 04: p16/Ki-67 and Hr-HPV test in ≥ CIN 2 lesions

This table summarizes the analysis for sensitivity, specificity, PPV and NPV with gold standard ≥ CIN2 lesions for p16/Ki-67 and hr-HPV tests for abnormal Pap cases. The sensitivity of dual-stained cytology and hr-HPV DNA testing was 96.8% for ≥ CIN2 in all abnormal groups. The specificity of dual-stained cytology was 70.2% for whereas hr-HPV test had specificity of 55.8% (table 04).

Specificity for dual stained cytology was higher than hr-HPV test. The positive predictive value for dual stained cytology was 61.2% whereas hr-HPV test had 51.7%. The positive predictive value for dual stained cytology was higher than hr-HPV test. The negative predictive value for dual stained cytology and hr-HPV was similar 97.6%. P value and 95% CI difference for both tests were not significantly significant (table 04).

PERFORMANCE OF P16/KI-67 AND HR-HPV IN DETECTION OF ≥ CIN3 LESIONS:

	Sensitivity %(95%CI)	**Specificity %(95%CI)**	**PPV %(95%CI)**	**NPV %(95%CI)**
Hr-HPV test	100	47.4	31	100
P16/Ki-67 ≥ 1 positive cell	100	59.2	36.7	100
P value	1.000	0.186	0.534	1.000
95% CI Difference	-14 to 14	-29 to 05	-12 to 03	-13 to 13

Table 05: p16/Ki-67 and Hr-HPV test in≥ CIN 3 lesions

This table summarizes the analysis for sensitivity, specificity, PPV and NPV with gold standard ≥ CIN3 lesions for p16/Ki-67 and hr-HPV tests for abnormal Pap cases. The sensitivity and negative predictive value for p16/Ki-67 dual immunocytochemistry and high-risk HPV test was 100% for ≥ CIN3 lesions. Specificity of p16/Ki-67 was 59.2 % and specificity of high risk HPV test was 47.4 %.

The positive predictive value for dual immunocytochemistry was 36.7 % whereas 28.6% for hr-HPV test. The specificity and positive predictive value of dual stained cytology were slightly higher than that of hr-HPV test. P value and 95% CI difference for both tests were not significantly significant (table 05).

THRESHOLD FOR POSITIVE IMMUNOCYTOCEMISTRY IN≥ CIN2 LESIONS:

The presence of one or more cervical epithelial cells with co expression of brown cytoplasmic P16 immunostaining and red nuclear Ki-67 immunostaining within the same cell is regarded as a positive CINtec PLUS test result. However, we also looked at performance of dual immunocytochemistry with a threshold of positivity for more than 10 cells(68).

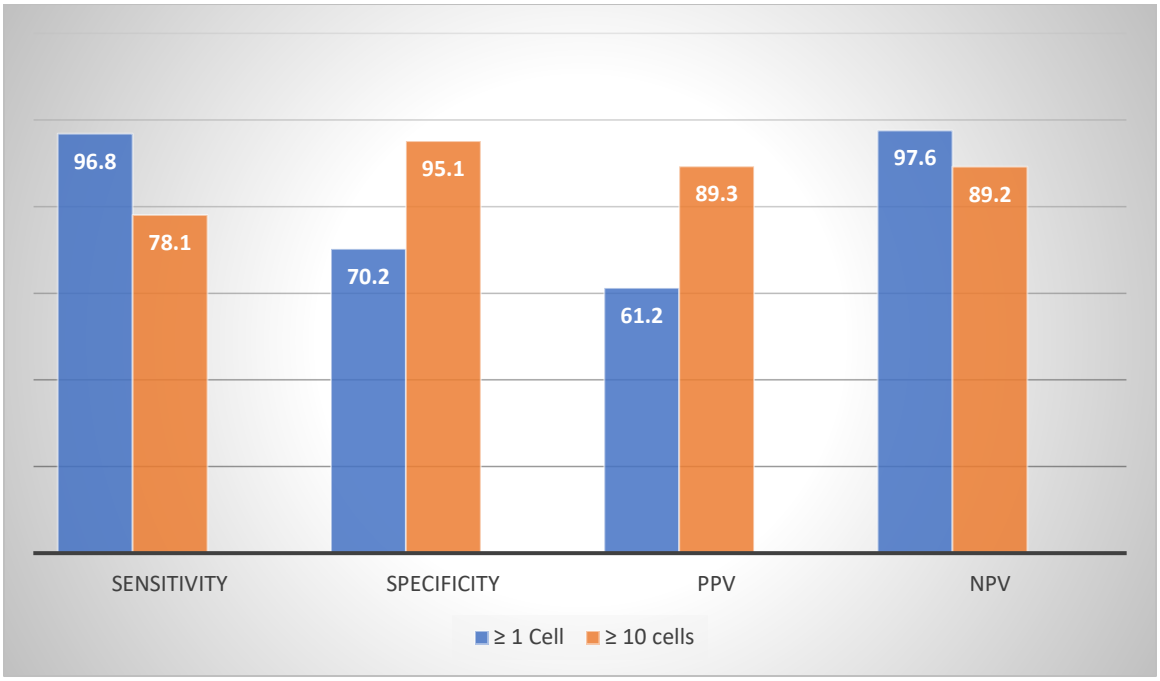


Figure 15: Performance of p16/Ki-67 immunocytochemistry with different thresholds in detection of ≥CIN2 lesions

Increase in threshold showed higher specificity (95.1 vs 70.2) and positive predictive value (89.3 vs 61.2) but there was decrease in sensitivity (78.1 vs 96.8) and negative predictive value (89.2 vs97.6).

	Sensitivity %(95%CI)	Specificity %(95%CI)	PPV %(95%CI)	NPV% (95%CI)
Hr-HPV test	96.8	55.8	51.7	97.6
P16/Ki-67≥ 10 positive cells	78.1	95.1	89.3	89.2
P value	0.026	<0.001	0.001	0.132
95% CI difference	2 to 34	25 to 52	20 to 54	-07 to 17

Table 06： p16/Ki-67(≥10 cells) and Hr-HPV test in≥ CIN 2 lesions

This table summarizes the statistical difference for sensitivity, specificity, PPV and NPV. P value and 95% CI for sensitivity, specificity and positive predictive values were statistically significant. Negative predictive value was not statistically significant (table 06).

THRESHOLD FOR POSITIVE IMMUNOCYTOCEMISTRY IN ≥CIN3+ LESION:

The diagnostic capability of dual stained cytology for ≥ CIN 3 lesions with positivity for more than 10 cells showed higher specificity (82.7 vs 59.2) and higher positive predictive value (53.6 vs 36.7) but there was decrease in sensitivity (83.8 vs 100) and negative predictive value (100 vs 95.4).

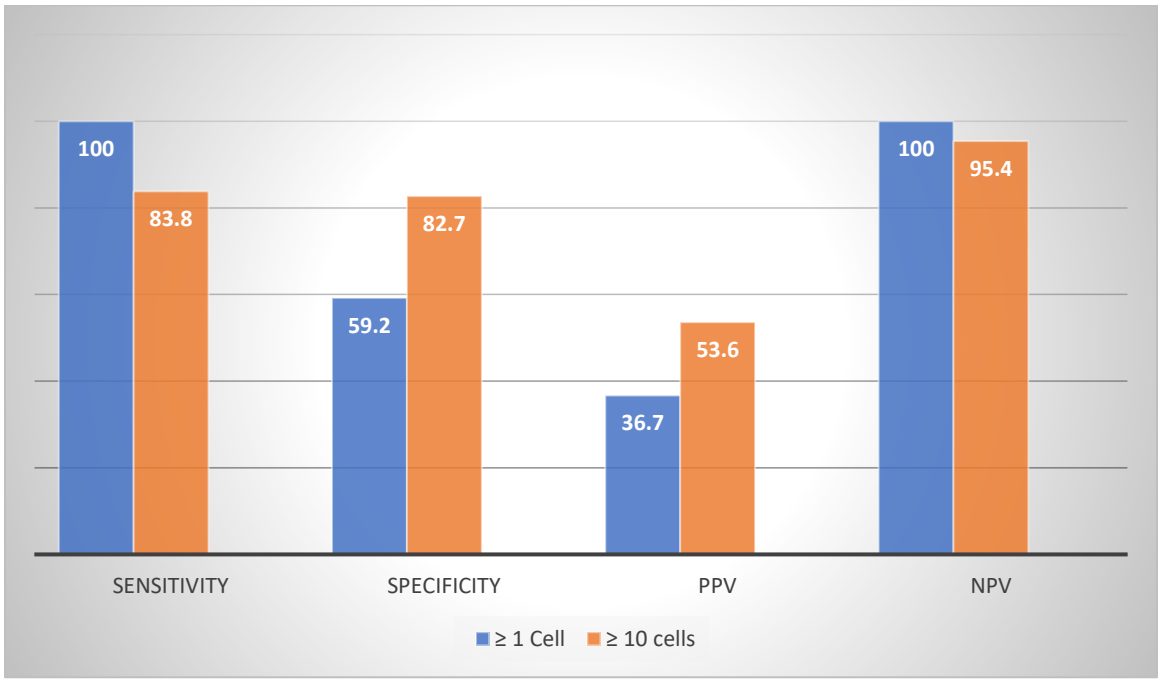


Figure 16: Performance of p16/Ki-67 immunocytochemistry with different thresholds in detection of ≥ CIN3 lesions

	Sensitivity %(95%CI)	Specificity %(95%CI)	PPV %(95%CI)	NPV %(95%CI)
Hr-HPV	100	47.3	31	100
P16/Ki-67≥ 10 positive cells	83.8	82.7	53.6	95.4
P value	<0.001	<0.001	0.051	<0.001
95% CI difference	57 to 90	19 to 50	00 to 45	75 to 93

Table 07: p16/Ki-67 (≥10 cells) and Hr-HPV test in ≥ CIN 3 lesions

This table summarizes the analysis for sensitivity, specificity, PPV and NPV. P value and 95% CI for sensitivity, specificity and negative predictive values were statistically significant. Positive predictive value was not statistically significant (table 07).

PERFORMANCE OF P16/KI-67 AND HR-HPV TEST IN DETECTION OF ≥ CIN2 LESIONS IN WOMEN <30 YEARS:

	Sensitivity %(95%CI)	Specificity %(95%CI)	PPV %(95%CI)	NPV %(95%CI)
Hr-HPV test	100	45.5	12.1	100
P16/Ki-67 ≥1 positive cell	100	36.4	14.3	100
P value	1.000	0.301	0.737	1.000
95% CI difference	-14 to 14	-26 to 08	-10 to 15	-13 to 13

Table 08: p16/Ki-67 and Hr-HPV test in ≥CIN 2 lesions in women <30 years

This table summarizes the analysis for sensitivity, specificity, PPV and NPV with gold standard ≥ CIN2 lesions for p16/Ki-67 and hr-HPV tests for abnormal Pap cases in women < 30 years. There were 12 women less than 30 years of age and 82 women more than 30 years of age. The sensitivity and negative predictive values of hr-HPV and dual stained cytology were similar 100%. The specificity of hr-HPV was 45.5% and positive predictive value 12.1%. Sensitivity, specificity and negative predictive value and positive predictive value 95% CI difference and P value were not statistically significant (table 08).

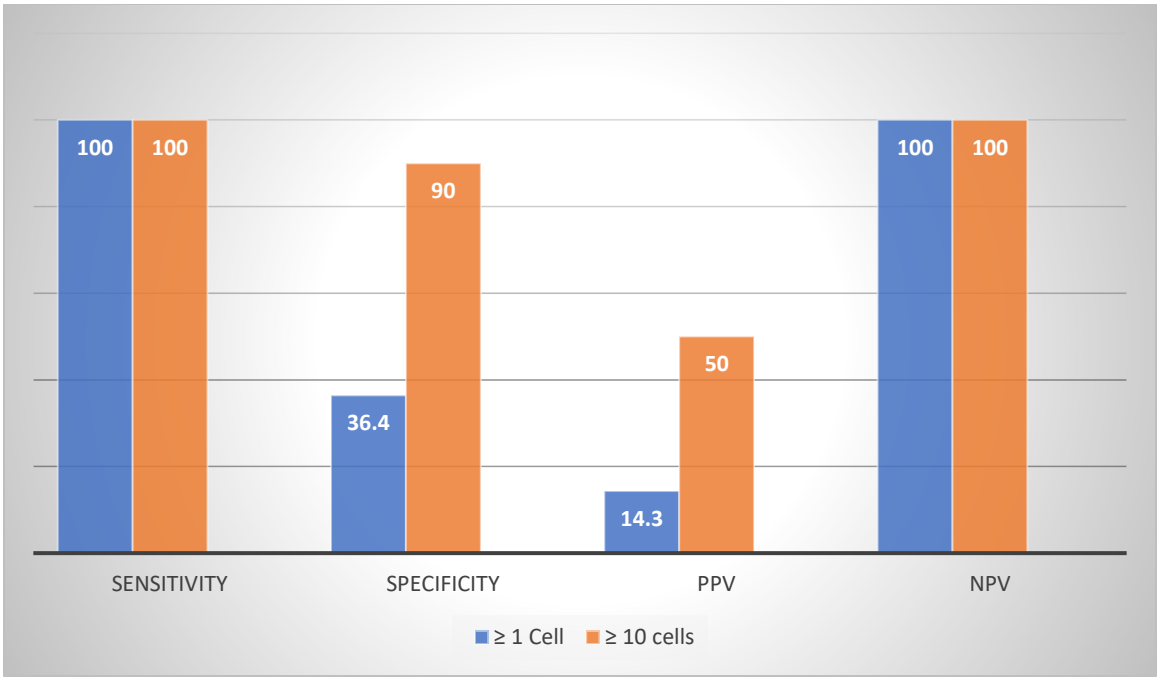


Figure 17: Performance of p16/Ki-67 immunocytochemistry with different thresholds to detect ≥ CIN2 lesions in women <30 years

The dual immunocytochemistry showed, specificity of 36.4% and positive predictive value of 14.3% when a score of one or more cells was the positive threshold. Both tests had low specificity and positive predictive value. When the threshold was increased to more than ten cells there was significant increase in specificity (90 vs 30.6) and positive predictive value (50 vs 14.3). P value and 95% CI for specificity and positive predictive value were statistically significant. Sensitivity and negative predictive values were not statistically significant (table 09).

PERFORMANCE OF P16/KI-67 AND HR-HPV TEST IN DETECTION OF ≥ CIN2 LESIONS IN WOMEN LESS THAN 30 YEARS.

	Sensitivity %(95%CI)	**Specificity %(95%CI)**	**PPV %(95%CI)**	**NPV %(95%CI)**
Hr-HPV test	100	45.5	12.1	100
P16/Ki-67 >10 positive cells	100	90	50	100
P value	1.000	<0.001	<0.001	1.000
95% CI Difference	-14 to 14	30 to 58	17 to 58	-10 to 10

Table: 09: p16/Ki-67(≥10 cells) and Hr-HPV test in ≥ CIN 2 lesions in women <30 years

This table summarizes the analysis for sensitivity, specificity, PPV and NPV. P value and 95% CI for specificity and positive predictive values were statistically significant. sensitivity and negative predictive value was not statistically significant.

PERFORMANCE OF P16/KI-67 AND HR-HPV TEST IN DETECTION OF ≥ CIN2 LESIONS IN WOMEN MORE THAN 30 YEARS.

	Sensitivity %(95%CI)	Specificity %(95%CI)	PPV %(95%CI)	NPV %(95%CI)
Hr-HPV test	93.5	56.9	56.9	93.5
P16/Ki-67≥ 1 positive cell	93.5	76.5	70.7	95.1
P value	1.000	0.020	0.140	0.756
95% CI difference	-12 to 12	03 to 35	-04 to 31	-08 to11

Table10: P16/KI-67 and hr-HPV test in ≥ CIN2 lesions in women >30 years

This table summarizes the analysis for sensitivity, specificity, PPV and NPV for ≥ CIN2 lesions p16/Ki-67 immunocytochemistry and hr-HPV tests for women >30 years. The sensitivity of hr-HPV test and dual immunocytochemistry cytology were similar 100% in all 82 women >30 years of age. The negative predictive values of both hr-HPV test and dual stained cytology were relatively high (93.5 and 95.1). The specificity and positive predictive value for hr-HPV test were 56.9%. P value and 95% CI difference for specificity was statistically significant. Sensitivity, positive and negative predictive values were not statistically significant (table 10).

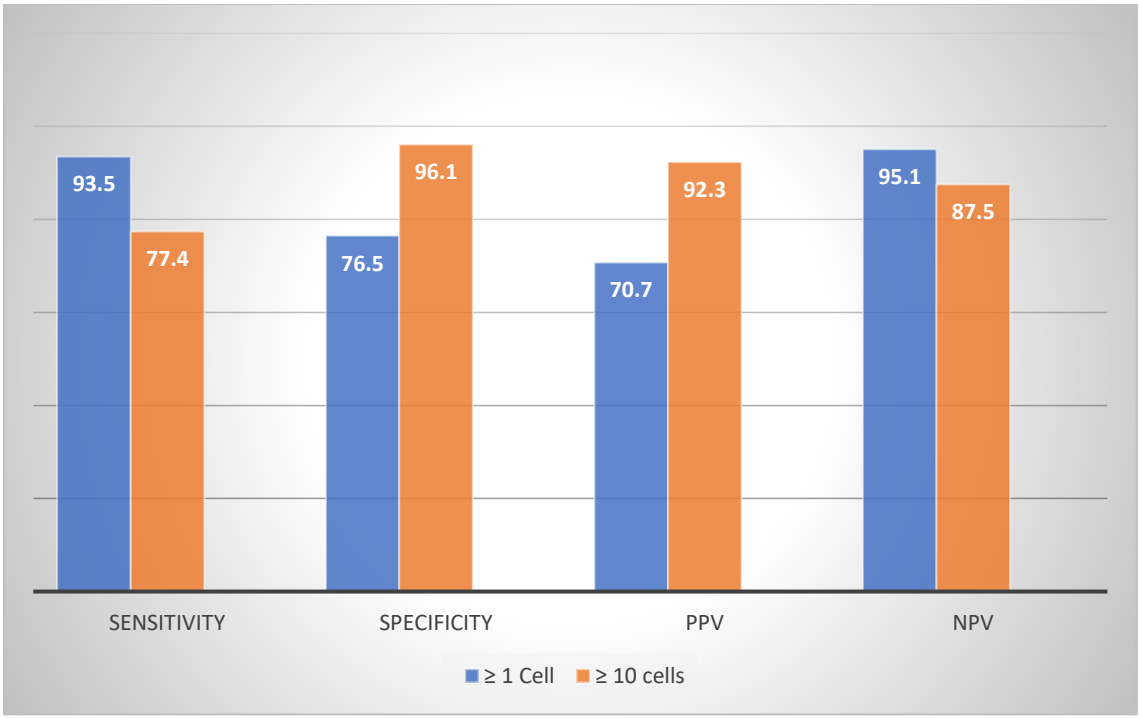


Figure 17: Performance of p16/Ki-67 immunocytochemistry with different thresholds to detect ≥ CIN2 lesions in women >30 years

The dual stained cytology showed, specificity of 76.5% and positive predictive value of 70.7% when the threshold was taken as ≥ 1 cell. When the threshold was more than ten cells, there was increase in specificity (96.1 vs 76.5) and positive predictive value (92.3vs 70.7).

	Sensitivity %(95%CI)	Specificity %(95%CI)	PPV %(95%CI)	NPV %(95%CI)
Hr-HPV	93.5	56.9	56.9	93.5
P16/Ki-67≥10 positive cells	77.4	96.1	92.3	87.5
P value	0.072	<0.001	0.001	0.342
95% CI difference	-33 to 01	26 to 52	19 to 51	-17 to 53

Table 11: P16/KI-67 (≥ 10 cells) and hr-HPV test in ≥ CIN2 lesions in women >30 years

This table summarizes the analysis for sensitivity, specificity, PPV and NPV. P value and 95% CI difference for specificity and positive predictive value were statistically significant. Sensitivity and negative predictive value was not statistically significant (table:11).

AGREEMENT BETWEEN HR-HPV TEST AND P16/KI-67:

The kappa for hr-HPV test to detect ≥ CIN2 lesions was 0.406. When > 1 cell was used as the threshold, the kappa was 0.559 but the agreement was increased to 0.754, when more than 10 cells were used as the threshold. Thus, the kappa agreement for dual immunocytochemistry for ≥ CIN2 lesions was higher when compared to hr-HPV test.

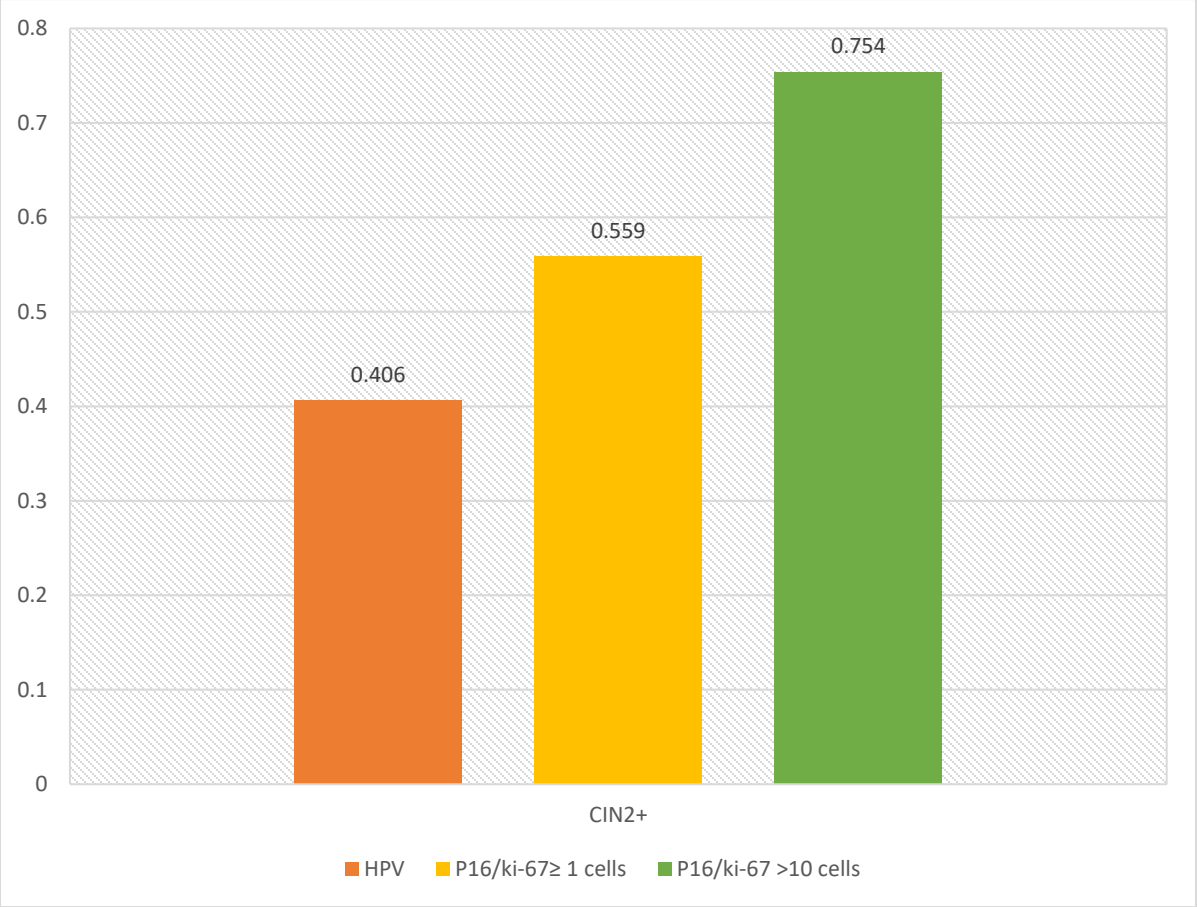


Figure 18: Agreement between p16/Ki-67 and hr-HPV test ≥ CIN2 lesions with different thresholds.

PERFORMANCE OF P16/KI-67 AND HR-HPV TEST IN DETECTION OF ≥ CIN2 LESIONS IN ASC-US.

	Sensitivity %(95%CI)	Specificity %(95%CI)	PPV %95%CI)	NPV %(95%CI)
Hr-HPV test	100	71.8	21.4	100
P16/Ki-67≥ 1 Positive cell	100	82.1	30	100
P value	1.000	0.280	0.631	1.000
95% CI difference	-48 to 48	-08 to 28	-27 to 44	-15 to 15

Table 12: p16/Ki-67 and hr-HPV in CIN ≥ 2 lesions for ASC-US

This table summarizes the analysis for sensitivity, specificity, PPV and NPV with gold standard ≥ CIN2 lesions for ASC-US group. Sensitivity and negative predictive value for p16/Ki-67 dual immunocytochemistry and hr-HPV test were similar (100%) to detect high grade lesions among ASCUS group. P16/Ki-67 specificity was 82 % and positive predictive value was 30%. Hr-HPV test specificity was 71.8% and positive predictive value was 21.4%. P value and 95% CI difference for both tests were not significantly significant (table 12).

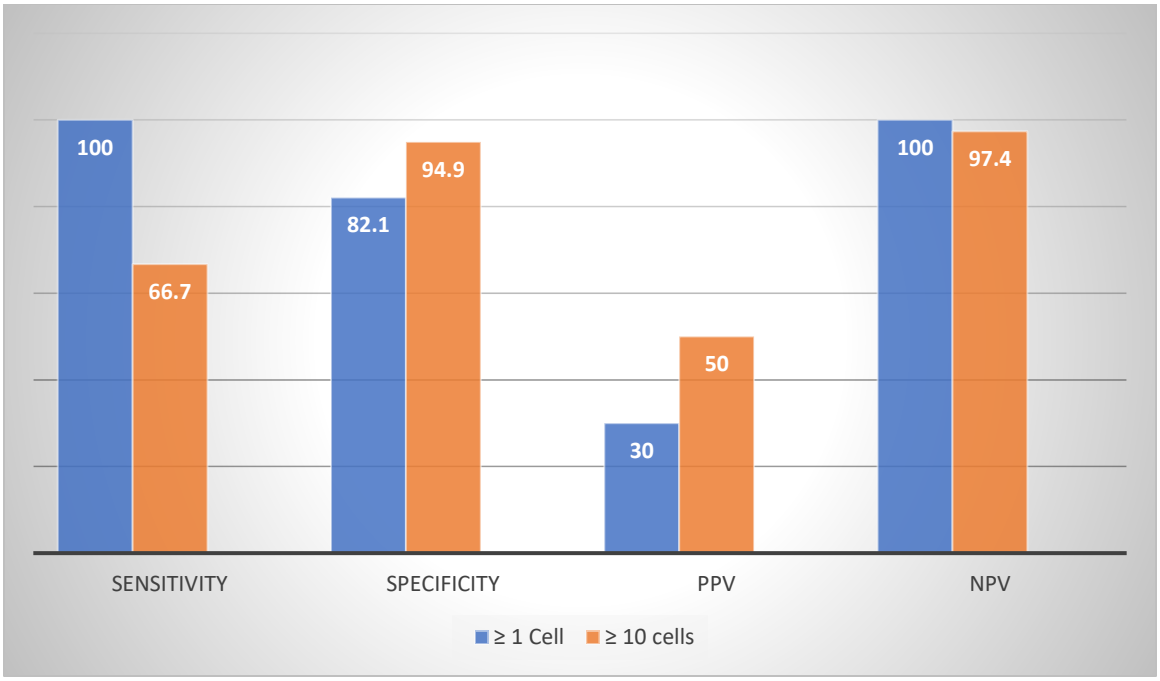


Figure 19: Performance of p16/Ki-67 immunocytochemistry with different thresholds to detect ≥ CIN2 lesions in ASC-US.

When the threshold was more than ten cells, there was increase in specificity (82.1 vs 94.9) and positive predictive value (30 vs 50) and decrease in sensitivity (100 vs 66.7) and negative predictive value (100 vs 97.4). Sensitivity, specificity and positive predictive value P value and 95% CI were not statistically significant. Negative predictive value was not statistically significant (table:13)

	Sensitivity %(95%CI)	Specificity %(95%CI)	PPV %(95%CI)	NPV %(95%CI)
Hr-HPV test	100	71.8	21.4	100
P16/Ki-67 ≥ 10 positive cells	66.7	94.9	50	97.4
P value	0.185	0.006	0.260	<0.001
95% CI difference	-16 to 30	07 to 38	-24 to 82	75 to 99

Table 13: p16/Ki-67(≥ 10 cells) and hr-HPV in CIN ≥ 2 lesions for ASC-US

This table summarizes the analysis for sensitivity, specificity, PPV and NPV. P value and 95% CI difference for specificity was statistically significant. Sensitivity positive predictive value and negative predictive value were not statistically significant (table:13)

PERFORMANCE OF P16/KI-67 AND HR-HPV IN DETECTION OF ≥ CIN2 LESIONS IN LSIL:

	Sensitivity %(95%CI)	Specificity %(95%CI)	PPV %(95%CI)	NPV %(95%CI)
Hr-HPV test	100	21.4	8.3	100
P16/Ki-67≥ 1 positive cell	100	64.3	28.6	100
P value	1.000	0.184	0.242	1.000
95% CI difference	-58 to 58	-15 to 31	-16 to 57	-35 to 35

Table 14: p16/Ki-67 and hr-HPV test in ≥ CIN2 lesions for LSIL

This table summarizes the analysis for sensitivity, specificity, PPV and NPV with gold standard ≥ CIN2 lesions for LSIL group. Sensitivity and Negative Predictive Value for p16/Ki-67 dual immunocytochemistry and hr-HPV test were similar (100%) to detect high grade lesions among LSIL group. P16/Ki-67 specificity was 64.3 % and positive predictive value was 28.6%. Hr-HPV test specificity was 21.4% and positive predictive value was 8.3%. P value and 95% CI difference for sensitivity, specificity, PPV and NPV values were not statistically significant. (table:14)

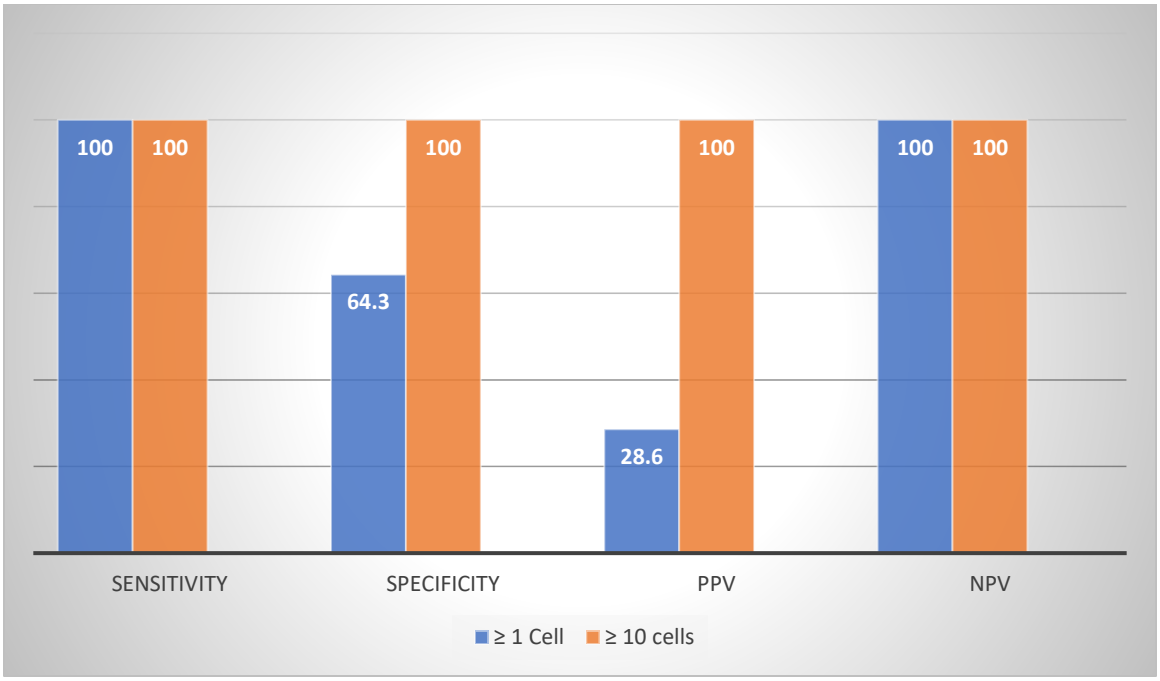


Figure 20: Performance of p16/Ki-67 immunocytochemistry with different thresholds to detect ≥ CIN2 lesions in LSIL.

When the threshold was more than ten cells, there was increase in specificity (64.3 vs 100) and positive predictive value (28.6 vs 100) and similar in sensitivity (100) and negative predictive value (100).

	Sensitivity %(95%CI)	Specificity %(95%CI)	PPV %(95%CI)	NPV %(95%CI)
Hr-HPV test	100	21.4	8.3	100
P16/Ki-67 ≥ 10 positive cells	100	100	100	100
P value	1.000	0.407	0.937	1.000
95% CI difference	-58 to 58	-15 to 38	-42 to 46	-33 to 33

Table 15: p16/Ki-67(≥ 10 cells) and hr-HPV in CIN ≥ 2 lesions for in LSIL.

This table summarizes the analysis for sensitivity, specificity, PPV and NPV. P value and 95% CI difference for sensitivity, specificity, PPV and NPV values were not statistically significant.

MENOPAUSE STATUS:

There were 25 women in postmenopausal age group. The sensitivity and negative predictive value for hr-HPV test and dual stained cytology were similar 100%. The specificity for hr-HPV test was 58.3 and PPV was 72.5. Dual stained cytology had specificity of 66.7% and PPV 76.5% when cutoff was one or more cells but with cutoff of more than 10 cells showed 100 % specificity and PPV for ≥ CIN2 lesions.

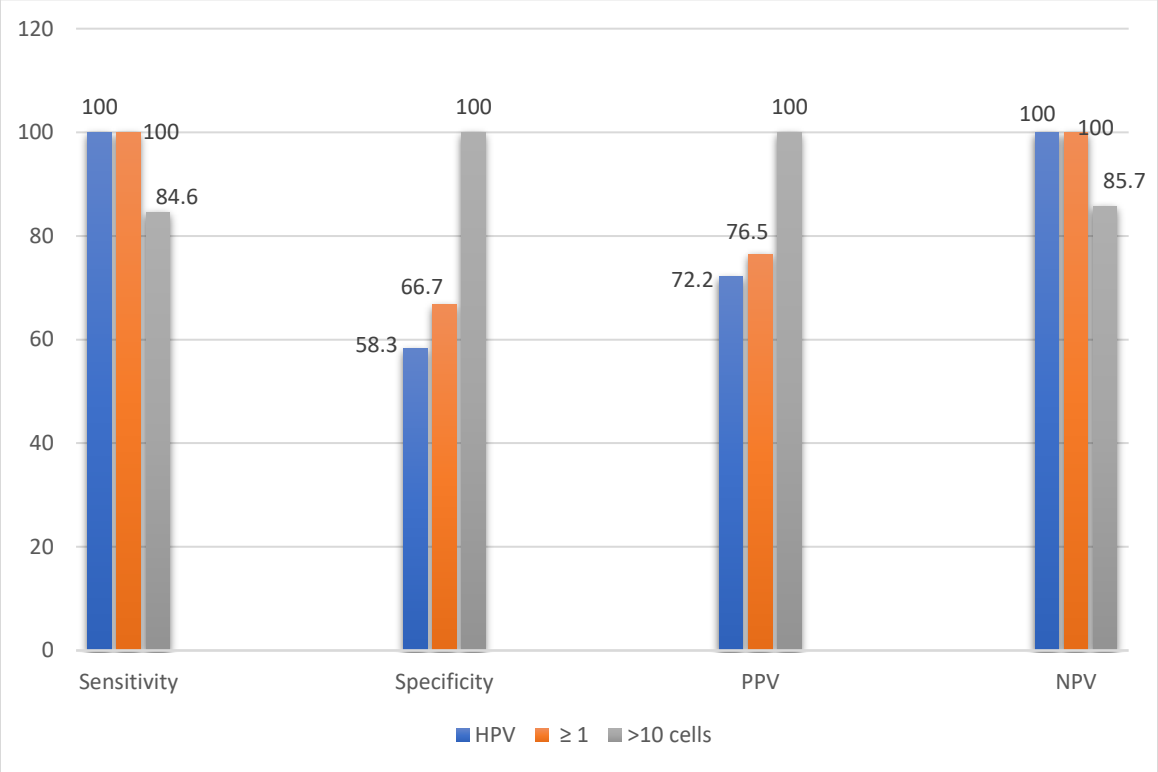


Figure 18: Performance of p16/Ki-67 immunocytochemistry and hr-HPV test to detect ≥ CIN2 lesions in postmenopausal women.

There were 69 women in reproductive age group. The sensitivity for both tests were similar (89%). The negative predictive value for hr-HPV test was 93.1 and p16/Ki-67 was 94.6%. The specificity for hr-HPV test was 48.3 and PPV was 22.5. p16/Ki-67 Dual immunocytochemistry with more than ten cells cutoff showed significantly improved specificity (93.9 vs 70), PPV (82.4 vs 53.1) and decrease in sensitivity (73.7 vs 89.5) and NPV (90.2 vs 94.6%) as compare to a threshold of one or more cells.

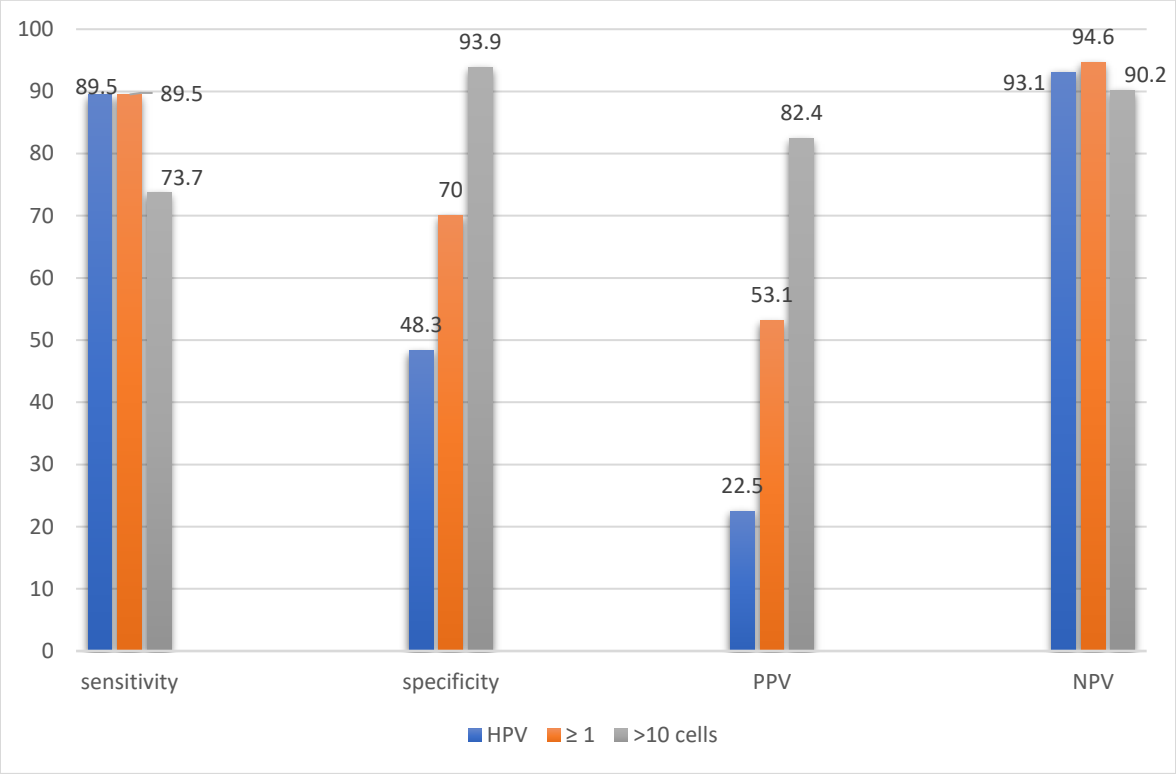


Figure 19: Performance of P16/Ki-67 immunocytochemistry and hr-HPV test in detection of ≥ CIN2 lesions in reproductive age group women

Images:

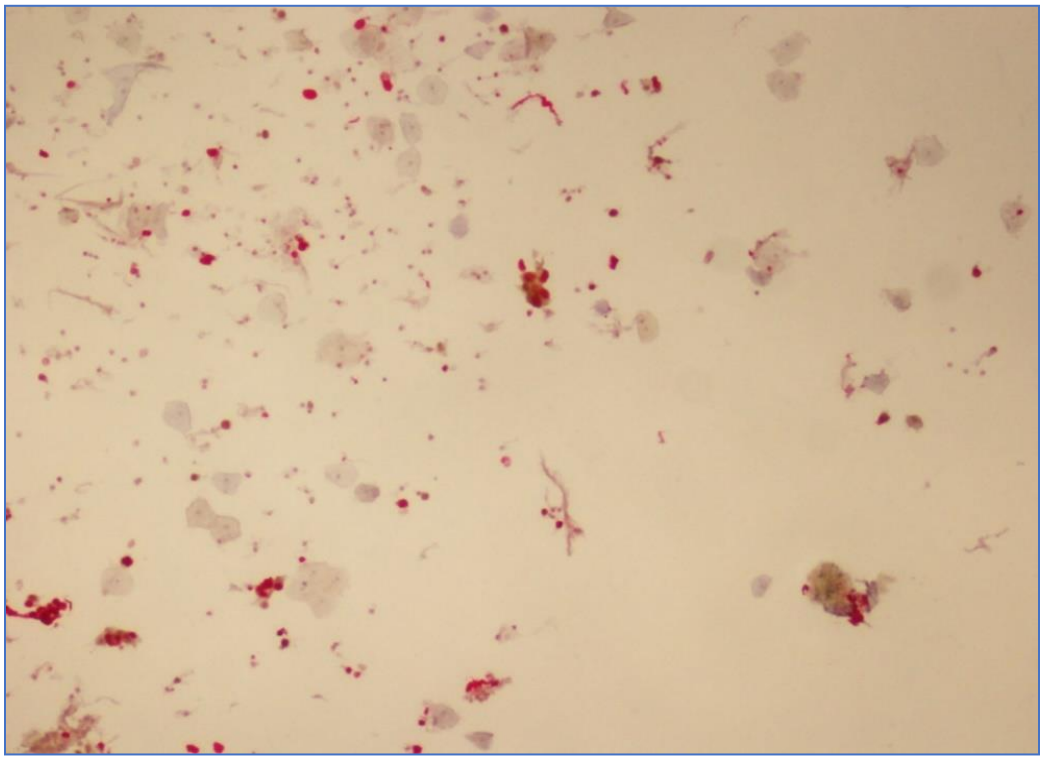


Figure 20: Positive control of p16/Ki-67 dual ICC at 40x

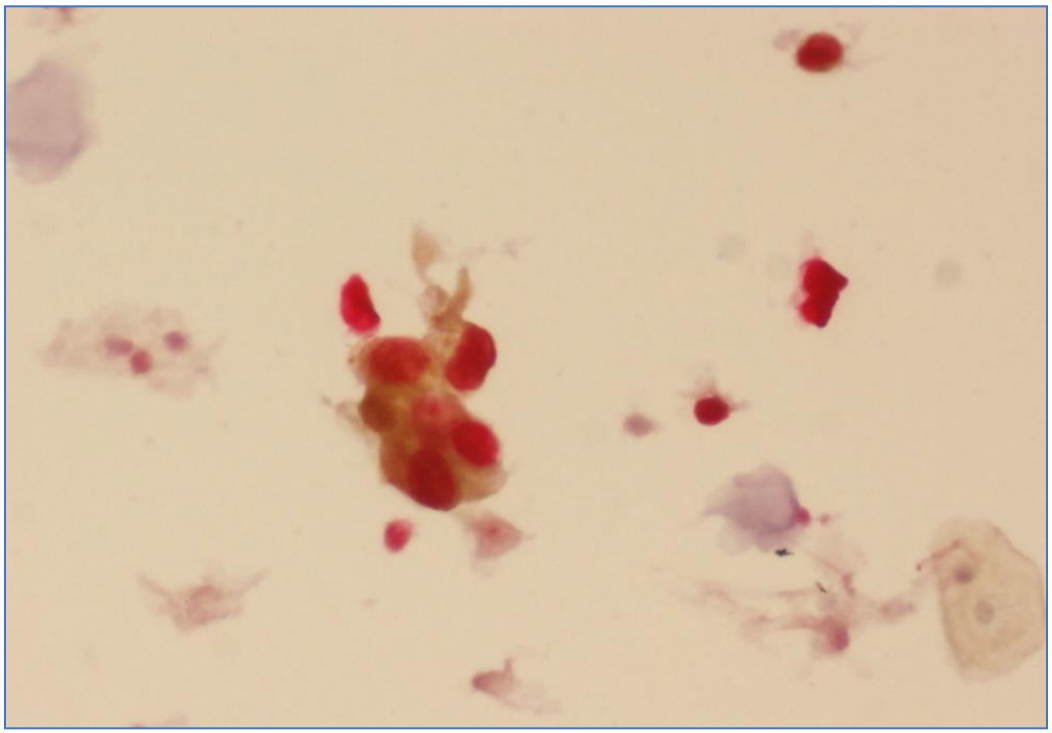


Figure 21: Positive control of p16/Ki-67 dual ICC at 200x.

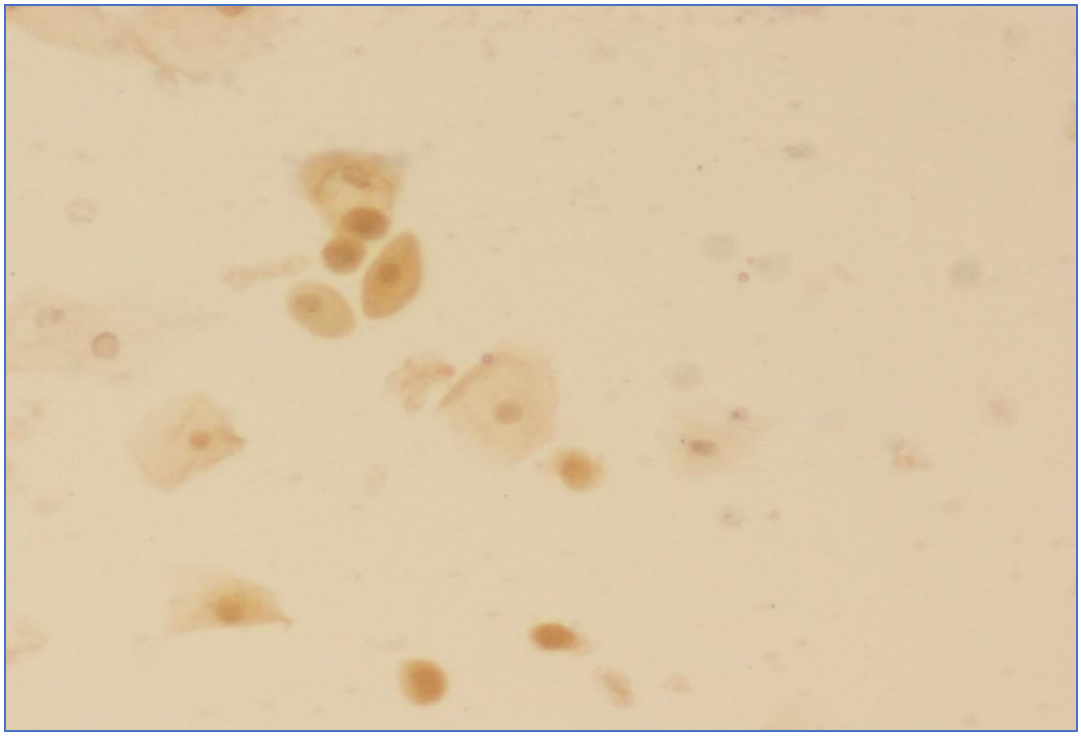


Figure 22: Negative control of p16/Ki-67 dual ICC at 200x

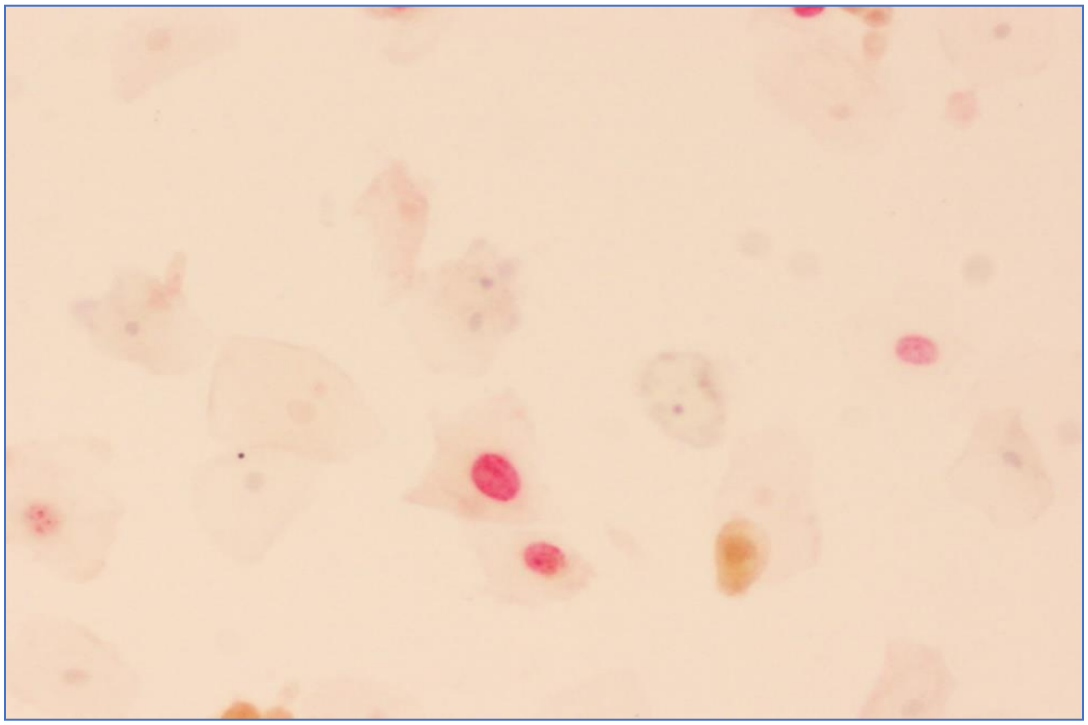


Figure 23: Negative control of p16/Ki-67 dual ICC at 200x

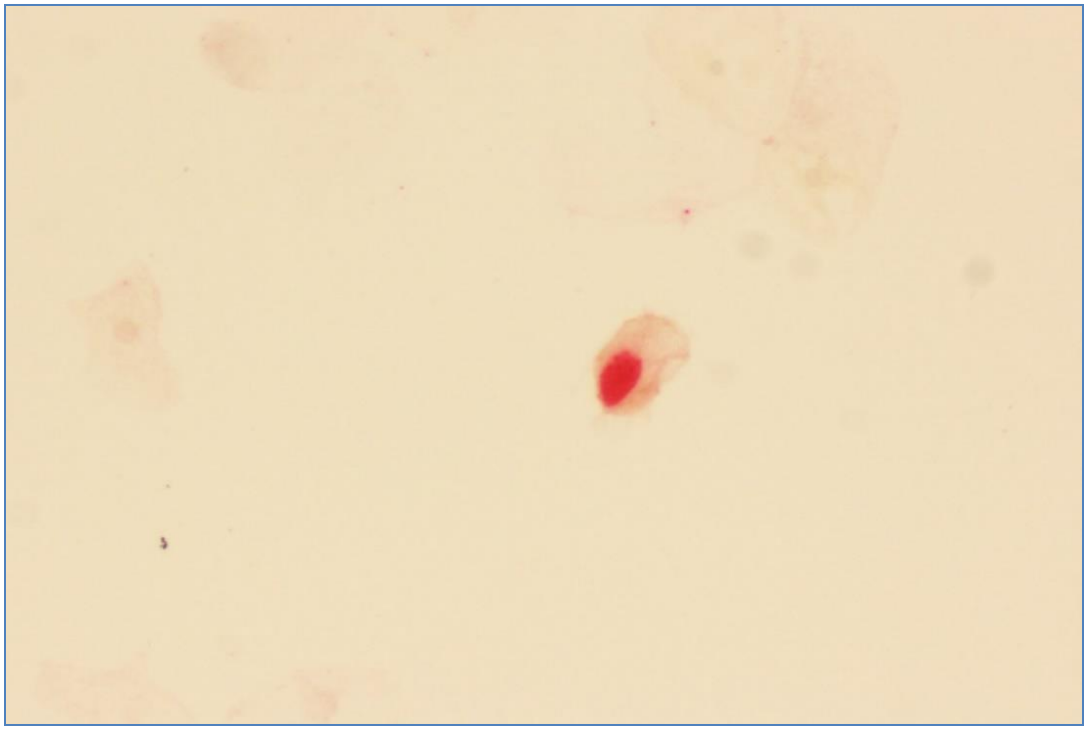


Figure 24: P16/ki67 dual ICC- ASCUS at 200x.

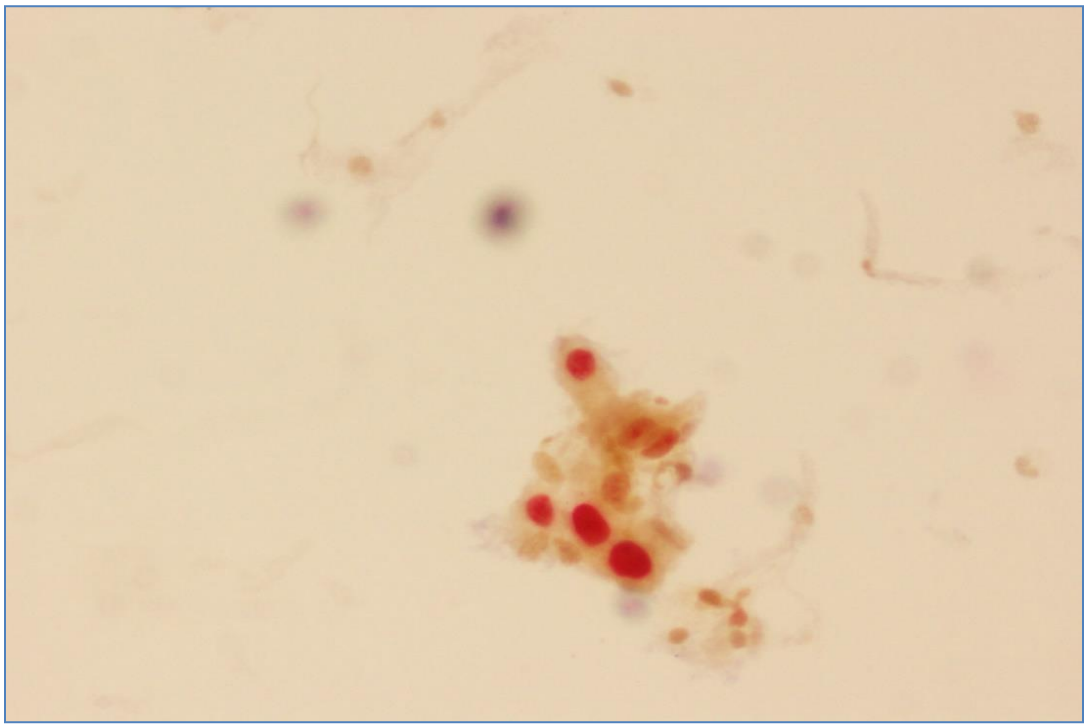


Figure 25: P16/Ki-67 dual immunocytochemistry -LSIL at 200x

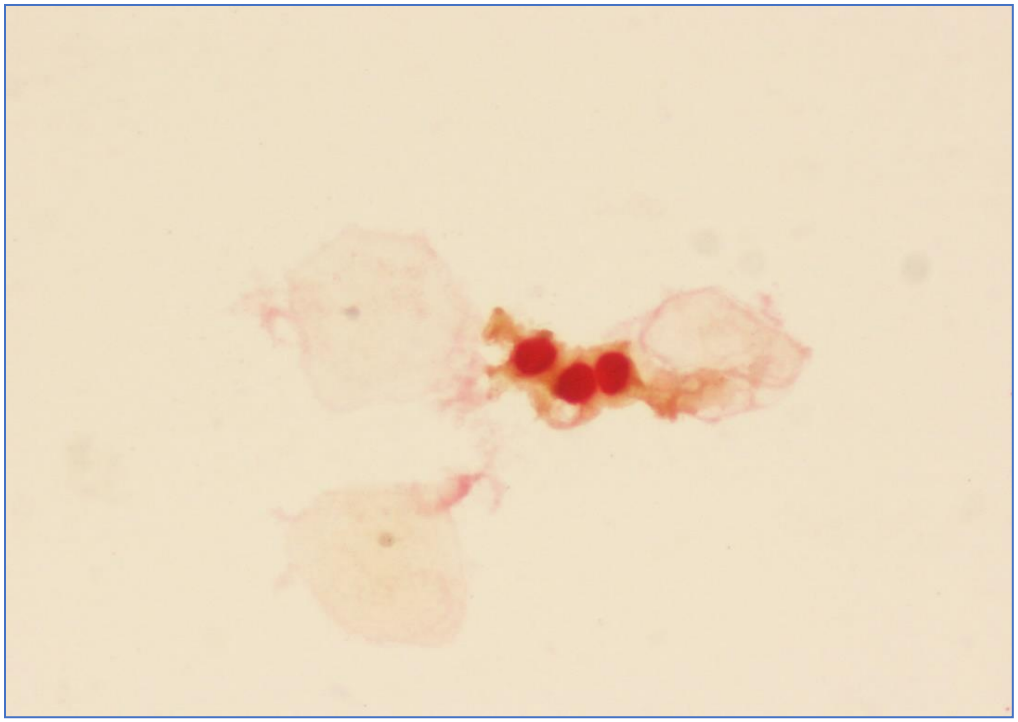


Figure 27: P16/Ki-67 dual ICC- ASC-H at 200x

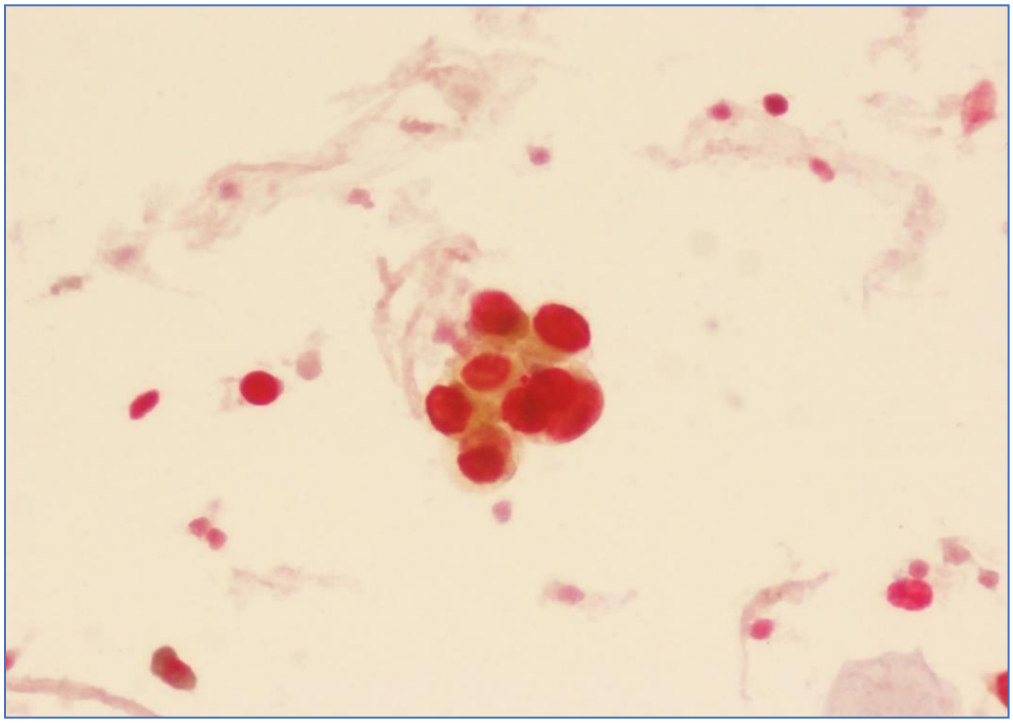


Figure 27: P16/Ki-67 dual ICC - HSIL at 200x

DISCUSSION

Cervical cancer is still one of the major causes of death worldwide, particularly more common in developing countries. Pap smear is still one the most efficient tests used for screening of cervical carcinoma in many countries. The disadvantage of using Pap smear is that it has low sensitivity and relatively high specificity. Since the awareness about association between cervical carcinoma and human papilloma virus, high risk HPV DNA test was implemented as a potential screening test to improve the sensitivity and to identify 13 high risk types of HPV.

This test had shown good sensitivity when compared to Pap smear screening but it has low specificity. Presence of persistent hr- HPV was associated with more risk of developing precancerous cervical lesion followed by invasive malignancy due to dysregulation of cell cycle by incorporation oncogenic viral proteins into the host genome.

This hr-HPV test identifies all women infected with hr- HPV types, irrespective of the nature of infection. Most of the infections which were transient in nature resulted in clearance of high risk infection within 1-2 years. However, the triage of minor cervical cytology abnormalities is still not clear. If hr-HPV test is positive, then such patients are referred for colposcopy guided biopsy to rule out any precancerous or cancerous lesion. Since hr-HPV test has low specificity, particularly in the younger age (<30 years), there is an increased number of unnecessary referral to colposcopy which is associated with more morbidity. This also discourages the women from participating in screening programs, due to multiple repeated tests.

Thus, it was important to find out a better triaging test for hr-HPV positive women or women with minor cytological abnormalities to reduce the number of false positive cases and thereby to reduce the number of unwanted colposcopies and biopsies. One of the relatively recently introduced triage system for these cases is immunocytochemistry p16/Ki-67. p16 is a protein which triggers the cell cycle arrest under normal physiological state and Ki-67 indicates proliferating index of a cell. Co-expression of both p16 and Ki-67 in a same cell indicates dysregulated cell cycle due to incorporation viral oncoprotein into the host genome suggestive of an underlying high-grade lesion.

Our study is a prospective diagnostic study which evaluated performance of dual stained immunomarker p16/ki-67 with HC2 HPV assay in cervical screening for detection of high grade cervical intraepithelial lesion in abnormal Pap smears. This study included 42 ASC-US, 16 LSIL, 14 ASC-H and 22 HSIL cases of abnormal Pap smears from August 2016 to March 2017 for analysis.

The median age was 42.73 years (standard deviation of 11.4 years) for abnormal pap smears and was comparable to median age of 41 (Korolczuk et al, 2015). ASC-US was the most common group in the study. Nayar et al in the Bethesda system for reporting cervical cytology shown that ASC-US most is the most common abnormal cytology group.

Performance of p16/Ki-67 and hr-HPV test:

In our study the performance of dual immunocytochemistry for high grade (≥ CIN2) lesion was as follows: sensitivity 96.8%, negative predictive value 97.6%, specificity 70.2% and positive predictive value of 61.2%. Thus, for detecting high grade lesions (≥ CIN2) among all abnormal Pap smears, sensitivity and negative predictive value of dual immunocytochemistry was similar to HC2 HPV DNA testing, but with improved specificity and positive predictive values.

These results were comparable to You-Lin Qiao et al study (71). You-Lin Qiao et al in 2015performed cytology-based screening in total population of1290 women including pre -cancerous and cancerous lesions in china. You-Lin Qiao et al highlighted performance of p16/Ki-67 dual immunocytochemistry as following; sensitivity was 90.2% (94.4% hr- HPV test), specificity was 79.5% (76.9% for hr-HPV test), positive predictive value was 49.2% (47.1 for hr- HPV test), and negative predictive value was 97.6% (98.4% for hr- HPV test). You-Lin Qiao et al study highlighted high sensitivity and negative predictive value for hr-HPV test as compare to dual immunocytochemistry whereas our study showed similar results for sensitivity and negative predictive value for both tests (Table:10) The present study showed better specificity and positive predictive value for dual immunocytochemistry as compared to hr-HPV test which is similar to You-Lin Qiao et al study results.

	Our study: N=94 P16/Ki-67	You-Lin Qiao et al, N=1079 p16/Ki-67	Our study: N=94 Hr-HPV	You-Lin Qiao et al, N=1079 Hr-HPV
Sensitivity	96.8%	90.9%	96.8%	94.4%
Specificity	70.2%	79.5%	55.8%	76.9%
PPV	61.2%	49.2%	51.7%	47.1%
NPV	97.6%	97.6%	97.6%	98.4%

Table 16: Comparison of performance of p16/Ki-67 vs hr-HPV test in our study with other study

A few studies have highlighted the difference between the performance of dual immunocytochemistry for less than 30 and more than 30 years of age (Table 11).Bergeron et al showed performance of p16/Ki-67 in LSIL for women < 30 years and >30 years as follows: sensitivity(84.6% vs 86.5%), specificity (50%vs56%), PPV (23.2vs29.4%) and NPV (94.8 vs 95.1) whereas Peter Ziemke 2017 study results were following, sensitivity(81.6 vs 84.6), specificity(61.1 vs 75.7), PPV(42.5 vs 52.8) and NPV (90.4 vs 93.8%). Above studies show that performance of p16/Ki-67 for women more 30 years was better as compared to women less than 30 years.

Table:17: p16/Ki-67 immunocytochemistry and age dependency for LSIL cytology in different studies

Author	Age	number	Sensitivity	Specificity	PPV	NPV
Bergeron, 2015	<30 years	172	84.6	50	23.2	94.8
	>30 years	212	86.5	56	29.4	95.1
Peter Ziemke, 2017	<30 years	146	81.6	61.1	42.5	90.4
	>30 years	185	84.6	75.7	52.8	93.8

Table 18: Comparison of performance of p16/Ki-67(threshold of ≥ 1 cell) vs hr-HPV test in less than 30 years and more than 30 years in our study

	<30 years P16/Ki-67	>30 years P16/Ki-67	<30 years Hr-HPV test	>30 years Hr-HPV test
Sensitivity	100	93.5	100	93.5%
Specificity	36.4	76.5	45.5	56.9
PPV	14.3	70.7	12.1	56.9
NPV	100	95.1	100	93.5

In our study, performance of dual immunocytochemistry was following between less than 30 years and more than 30 years: sensitivity (100 vs 93.5), specificity (36.4 vs 76.5), positive predictive value (14.3 vs 70.7), negative predictive value (100 vs 95.1) The specificity and positive predictive value were much better for women more than 30 years as compared` to less than 30 years. Bergeron, 2015 and Peter Ziemke, 2017 also arrived at similar results, though they had looked only at LSIL.

Peter Ziemke in 2017, proposed that false positivity in older women is less as compared to women less than 30, due to the longer infective period in older women. It could also be a good indicator of reparative and productive function of younger women. Peter Ziemke further demonstrated in the same study, increased specificity and predictive value when cutoff for dual immunocytochemistry positivity was more than 10 cells. Using a score of 10 P16/Ki-67 marked cells as a positive result instead of 1 led to significantly higher specificity (89% vs 70.2%) and positive predictive value (55.7% vs 46.7%) among women in LSIL group.

In our study, including all age groups, when we increased the cut off for positive interpretation to 10 cells, there was significantly increase in specificity (90.2 % vs 70.2%) and positive predictive value (89.3% vs 61.2%) whereas sensitivity (96.8 vs 78.1) and negative predictive value (97.6% vs 89.2%) relatively decreased.

In women less than 30 years, specificity was 36.4% and positive predictive value was 14.3% with a threshold of ≥1cell. When we used cutoff of more than 10 cells, the specificity (90% vs 36.4%) and positive predictive value (50%vs14.3) increased significantly with no significant change in sensitivity and negative predictive value. This was due to reduction in the false positivity. Since the number of women <30 years in our study was low, statistical significance cannot be demonstrated. However, results show that if cut off was increased to more than 10 cells, number of false positive cases were reduced, resulting in increased specificity and positive predictive value.

In women more than 30 years, there was increase in specificity (96.1 vs 76.5) and positive predictive value (92.3 vs 70.7) but decrease in sensitivity (77.4 vs 93.5) and negative predictive value (87.5 vs 95.1).

Thus, increasing the cut off for dual immunocytochemistry, resulted in significantly increased specificity and positive predictive value and relative decrease in sensitivity and negative predictive value. These results were similar to that of Peter Ziemke, though he has included only the LSIL cases.

Performance of p16/Ki-67 (threshold of ≥ 1 cell) and hr-HPV among low grade cytological abnormalities:

We further assessed the performance of the dual immunocytochemistry and hr-HPV test among LSIL group, which was as follows: sensitivity (100% vs100%), negative predictive value (100% vs 100%), specificity (64.3%vs 26%) and positive predictive value (28.6% vs 09.09%) for high grade lesion (≥ CIN2). Hence, sensitivity and negative predictive value were similar (100%) for dual immunocytochemistry and hr-HPV test. However, dual immunocytochemistry showed better specificity and positive predictive value as compared to hr-HPV test. Our study results were comparable to Bergeron et al study results.

	Our study N=16 P16/Ki-67	Bergeron, 2015 N=384 P16/Ki-67	Our study Hr-HPV	Bergeron, 2015 Hr-HPV
Sensitivity	100%	85.7%	100%	98.4%
Specificity	64.3%	78.7%	26%	15.6%
PPV	28.6%	16.3%	09.09%	18.6%
NPV	100%	99.7%	100%	98%

Table 19: Comparison of performance of p16/Ki-67and hr-HPV test in LSIL

Bergeron et al and a few other studies, also assessed the performance of p16/ki-67 immunocytochemistry marker in ASC-US In our study, performance of p16/Ki-67 dual immunocytochemistry results among ASCUS group as compared to hr-HPV test was follows: sensitivity (100% vs 100%), NPV (100% vs 100%), specificity (82.1% vs 71.8%) and PPV (30.1% vs 21.4%) for high grade lesions (≥ CIN2). Present study showed that sensitivity and NPV were similar (100%) for dual immunocytochemistry and hr-HPV. However, dual immunocytochemistry showed better specificity and PPV as compare to hr-HPV test. Our study results were comparable to Bergeron et al study results.

	Our study N=42 P16/Ki-67	Bergeron, 2015 N=427 P16/Ki-67	Our study N=42 Hr-HPV	Bergeron, 2015 N=427 Hr-HPV
Sensitivity	100%	94.4%	100%	100%
Specificity	82.1%	78.7%	71.8%	60.4%
PPV	30.1%	16.3%	21.4%	10%
NPV	100%	99.7%	100%	100%

Table 20: Comparison of performance of p16/Ki-67and hr-HPV test in ASCUS

In our study one case of ASC-H with negative hr-HPV and dual immunocytochemistry had CIN2 on biopsy. In the study by You-Lin Qiao et al, 21 cases of ≥ CIN2 (21/231) lesions, were p16/Ki-67 negative. It indicates that not all precancerous lesions progress to cervical cancer(71). There was evidence that approximately 40% of undiagnosed CIN-2 will regress over 2 years. This could possibly explain the negativep16/Ki-67 immunocytochemistry. This case was also negative for hr-HPV test. Marianne Waldstrom et al in 2012 shown that4 cases of CIN2+ (4/133) lesion, were negative for both hr-HPV test and p16/Ki-67. (72)

The kappa agreement for dual stained immunocytochemistry for CIN2+ was higher when compared to hr-HPV test (0.559 vs 0.406). When higher threshold of more than 10 cells is used the agreement was (0.754 vs 0.559), suggesting thatP16/ki-67 test could be a better test as compared to hr-HPV test.

The present study performed in 94 women with abnormal Pap cytology, showed similar sensitivity and negative predictive value but increased specificity and positive predictive value for dual immunocytochemistry as compared to hr-HPV test. This data is concordant with literature. Since p16/ki-67 has better specificity and positive predictive value than hr-HPV test, it can be used as a triage for hr-HPV positive women. The disadvantage is that when one cell is used as a cutoff for positive interpretation, the specificity and positive predictive value were less. This could be due to increased number of false positivity particularly in younger women. By increasing the cut off to at least 10 positive

cells, there was increased specificity and positive predictive value, However, with decrease in sensitivity and negative predictive value.

There are more alternative newer triage tests emerging and under study for hr-HPV positive women, to improve the predictive values of high grade lesions. Even though most of the other triage methods are not yet approved for screening program, few studies such as methylation assay based molecular technique are being extensively studied in the recent times. (73)

CONCLUSIONS

1. ASC-US was the most common intraepithelial lesion in all age groups.
2. 80% of women were symptomatic and 20% of women were asymptomatic.
3. Bleeding per vagina was the most common presenting symptom.
4. Sensitivity (96.8%) and negative predictive value (97.6%) for hr-HPV test and p16/Ki-67 dual immunocytochemistry (≥ 1 positive cell) were similar, but the latter test showed better specificity (70.2 vs 55.8) and positive predictive value (61.2 vs 51.7) for ≥ CIN 2 lesions. However, this is not statistically significant.
5. 20% cases showed false positivity for p16/Ki-67 dual immunocytochemistry and 30% for hr-HPV test.
6. A higher cut off of at least 10 positive cells gives a clinically and statistically higher specificity (CI: 25 to 52) and positive predictive value (CI: 20 to 54), while however, slightly decreased sensitivity and negative predictive value.
7. Since High risk HPV test has high sensitivity and negative predictive value while P16/Ki-67 dual immunocytochemistry (≥ 10 positive cells) has high specificity and positive predictive value, the latter can be recommended as an ancillary test in hr-HPV test positive women, to reduce the number of women going for colposcopy and biopsies.
8. Thus P16/Ki-67 dual immunocytochemistry can be used for risk stratification and also for appropriate management in patients with ASCUS and LSIL.

LIMITATIONS

Due to a delay in acquiring the CINtec plus kit for immunocytochemistry, the residual samples and premade smears were kept in the refrigerator for up to 100 days, which resulted in many paucicellular and inadequate smears, which had to be excluded from the study.

BIBLIOGRAPHY

1. Globocan 2012 - Home [Internet]. [cited 2016 Nov 14]. Available from: http://globocan.iarc.fr/Default.aspx

2. American College of Obstetricians and Gynecologists. Cervical cancer screening and prevention. Practice Bulletin No. 157. Obstet Gynecol. January 2016. 127:e1-20.

3. Sahasrabuddhe VV, Luhn P, Wentzensen N. Human papillomavirus and cervical cancer: biomarkers for improved prevention efforts. Future Microbiol [Internet]. 2011 Sep [cited 2016 Nov 14];6(9). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3809085/

4. Roelens J, Reuschenbach M, von Knebel-Doeberitz M, Wentzensen N, Bergeron C, Arbyn M. p16INK4a immunocytochemistry versus HPV testing for triage of women with minor cytological abnormalities: A systematic review and meta-analysis. Cancer Cytopathol. 2012 Oct 25;120(5):294–307.

5. White C, Bakhiet S, Bates M, Keegan H, Pilkington L, Ruttle C, et al. Triage of LSIL/ASC-US with p16/Ki-67 dual staining and human papillomavirus testing: a 2-year prospective study. Cytopathol Off J Br Soc Clin Cytol. 2016 Aug;27(4):269–76.

6. Williams J. On Cancer of the Uterus: Being the Harveian Lectures for 1886. London, UK: H. K. Lewis; 1888.

7. Broders AC. CARCINOMA IN SITU CONTRASTED WITH BENIGN PENETRATING EPITHELIUM. J Am Med Assoc. 1932 Nov 12;99(20):1670–4.

8. Reagan JW, Seidemann IL, Saracusa Y. The cellular morphology of carcinoma in situ and dysplasia or atypical hyperplasia of the uterine cervix. Cancer 1953;6:224–34.

9. Richart RM. A modified terminology for cervical intraepithelial neoplasia. Obstet Gynecol. 1990 Jan;75(1):131–3.

10. Wright TC, Schiffman M. Adding a test for human papillomavirus DNA to cervical-cancer screening. N Engl J Med. 2003 Feb 6;348(6):489–90.

11. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. Lancet Lond Engl. 2007 Sep 8;370(9590):890–907.

12. Wright TC. CHAPTER 3 Pathology of HPV infection at the cytologic and histologic levels: Basis for a 2-tiered morphologic classification system. Int J Gynecol Obstet. 2006 Nov 1;94:S22–31.

13. The 1988 Bethesda System for reporting cervical/vaginal cytological diagnoses. National Cancer Institute Workshop. JAMA. 1989 Aug 18;262(7):931–4.

14. The Bethesda System for Reporting Cervical/Vaginal Cytologic Diagnoses: Report of the 1991 Bethesda Workshop. The Bethesda System Editorial Committee - Journals - NCBI [Internet]. [cited 2017 Jul 28]. Available from: https://www.ncbi.nlm.nih.gov/labs/articles/1612572/

15. Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, et al. The 2001 Bethesda System: terminology for reporting results of cervical cytology. JAMA. 2002 Apr 24;287(16):2114–9.

16. Wright TC, Cox JT, Massad LS, Carlson J, Twiggs LB, Wilkinson EJ, et al. 2001 Consensus guidelines for the management of women with cervical intraepithelial neoplasia. J Low Genit Tract Dis. 2003 Jul;7(3):154–67.

17. Sauter JL, Powlovich L, St John T, Leiman G. Have the 2006 American Society for Colposcopy and Cervical Pathology (ASCCP) guidelines enriched the yield of high-grade squamous intraepithelial lesions? Diagn Cytopathol. 2015 Aug;43(8):673–5.

18. The American Society for Colposcopy and Cervical Pathology (ASCCP) guidelines - 2012.

19. Crum CP. Symposium Part 1. Should the Bethesda System terminology be used in diagnostic surgical pathology?: Point. Int J Gynecol Pathol 2003;22:5–12.

20. Davey DD, Neal MH, Wilbur DC, Colgan TJ, Styer PE, Mody DR. Bethesda 2001 implementation and reporting rates: 2003 practices of participants in the College of American Pathologists Interlaboratory Comparison Program in Cervicovaginal Cytology. Arch Pathol Lab Med. 2004 Nov;128(11):1224–9.

21. Woodhouse SL, Stastny JF, Styer PE, et al. Interobserver variability in subclassifi cation of squamous intraepithelial lesions: results of the College of American Pathologists Interlaboratory Comparison Program in cervicovaginal cytology. Arch Pathol Lab Med. 1999;123:1079–84.

22. Cox JT, Solomon D, Schiffman M. Prospective follow-up suggests similar risk of subsequent CIN 2 or 3 among women with CIN 1 or negative colposcopy and directed biopsy. Am J Obstet Gynecol. 2003;188:1406–12.

23. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. Lancet Lond Engl. 2007 Sep 8;370(9590):890–907.

24. zur Hausen H. The search for infectious causes of human cancers: where and why (Nobel lecture). Angew Chem Int Ed Engl. 2009;48(32):5798–808.

25. IARC (2007) Human Papillomaviruses. IARC, Lyon.

26. Muñoz N, Castellsagué X, de González AB, Gissmann L. Chapter 1: HPV in the etiology of human cancer. Vaccine. 2006 Aug 31;24 Suppl 3:S3/1-10.

27. Smith JS, Lindsay L, Hoots B, Keys J, Franceschi S, Winer R, et al. Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. Int J Cancer. 2007 Aug 1;121(3):621–32.

28. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer. 2002 May;2(5):342–50.

29. Blaustein's Pathology of the Female Genital Tract Sixth Edition, page no 201-202.

30. Burchell AN, Winer RL, de Sanjosé S, Franco EL. Chapter 6: Epidemiology and transmission dynamics of genital HPV infection. Vaccine. 2006 Aug 31;24 Suppl 3:S3/52-61.

31. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. Lancet Lond Engl. 2007 Sep 8;370(9590):890–907.

32. Wright TC, Kuhn L (2006) Immunosuppression and the cervix: human immunodeficiency virus (HIV). In: Jordan JA, Singer A (eds) The cervix. Blackwell, Malden, pp 450–517.

33. Datta SD, Koutsky LA, Ratelle S, Unger ER, Shlay J, McClain T, et al. Human papillomavirus infection and cervical cytology in women screened for cervical cancer in the United States, 2003-2005. Ann Intern Med. 2008 Apr 1;148(7):493–500.

34. Castle PE, Wacholder S, Sherman ME, Lorincz AT, Glass AG, Scott DR, et al. Absolute risk of a subsequent abnormal pap among oncogenic human papillomavirus DNA-positive, cytologically negative women. Cancer. 2002 Nov 15;95(10):2145–51.

35. Wheeler CM, Hunt WC, Schiffman M, Castle PE, Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesions Triage Study Group. Human papillomavirus genotypes and the cumulative 2-year risk of cervical precancer. J Infect Dis. 2006 Nov 1;194(9):1291–9.

36. e57d7b072cb4648b4c0e0f68091cbaf14911.pdf [Internet]. [cited 2017 Jul 28]. Available from: https://pdfs.semanticscholar.org/1fd3/e57d7b072cb4648b4c0e0f68091cbaf14911.pdf

37. Park TJ, Richart RM, Sun X-W et al (1996) Association between HPV type and clonal status of cervical squamous intraepithelial lesions (SIL). J Natl Cancer Instit 88(66):355–358.

38. Ueda Y, Enomoto T, Miyatake T, Ozaki K, Yoshizaki T, Kanao H, et al. Monoclonal expansion with integration of high-risk type human papillomaviruses is an initial step for cervical carcinogenesis: association of clonal status and human papillomavirus infection with clinical outcome in cervical intraepithelial neoplasia. Lab Investig J Tech Methods Pathol. 2003 Oct;83(10):1517–27.

39. Johnson LD (1969) The histopathological approach to early cervical neoplasia. Obstet Gynecol Surv 24:735–767.

40. Abeler VM, Holm R, Nesland JM, Kjørstad KE. Small cell carcinoma of the cervix. A clinicopathologic study of 26 patients. Cancer. 1994 Feb 1;73(3):672–7.

41. Fujiwara H, Mitchell MF, Arseneau J, Hale RJ, Wright TC. Clear cell adenosquamous carcinoma of the cervix. An aggressive tumor associated with human papillomavirus-18. Cancer. 1995 Nov 1;76(9):1591–600.

42. Kaku T, Kamura T et al (1997) Adenocarcinoma of the uterine cervix with predominantly villogladular papillary growth pattern. Gynecol Oncol 64(1):147–152.

43. Ferry JA, Scully RE. “Adenoid cystic” carcinoma and adenoid basal carcinoma of the uterine cervix. A study of 28 cases. Am J Surg Pathol. 1988 Feb;12(2):134–44.

44. Nanda K, McCrory DC, Myers ER, Bastian LA, Hasselblad V, Hickey JD, et al. Accuracy of the Papanicolaou test in screening for and follow-up of cervical cytologic abnormalities: a systematic review. Ann Intern Med. 2000 May 16;132(10):810–9.

45. The Bethesda System for Reporting Cervical Cytology - Definitions, | Ritu Nayar | Springer [Internet]. [cited 2017 Jul 28]. Available from: http://www.springer.com/in/book/9783319110738

46. Human papillomavirus testing for triage of women with cytologic evidence of low-grade squamous intraepithelial lesions: baseline data from a randomized trial. The Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesions Triage Study (ALTS) Group. J Natl Cancer Inst. 2000 Mar 1;92(5):397–402.

47. Jones BA, Novis DA. Cervical biopsy-cytology correlation. A College of American Pathologists Q-Probes study of 22 439 correlations in 348 laboratories. Arch Pathol Lab Med. 1996 Jun;120(6):523–31.

48. p16 Immunohistochemistry Interpretation by Nonpathologists as an Accurate Method for Diagnosing Cervical Precancer and Cancer [Internet]. [cited 2017 Jul 28]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5463559/

49. Mitchell MF, Tortolero-Luna G, Wright T, Sarkar A, Richards-Kortum R, Hong WK, et al. Cervical human papillomavirus infection and intraepithelial neoplasia: a review. J Natl Cancer Inst Monogr. 1996;(21):17–25.

50. Castle PE, Schiffman M, Wheeler CM, Solomon D. Evidence for frequent regression of cervical intraepithelial neoplasia-grade 2. Obstet Gynecol. 2009 Jan;113(1):18–25.

51. Romagosa C, Simonetti S, López-Vicente L, Mazo A, Lleonart ME, Castellvi J, et al. p16(Ink4a) overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. Oncogene. 2011 May 5;30(18):2087–97.

52. Weber JD, Jeffers JR, Rehg JE, Randle DH, Lozano G, Roussel MF, et al. p53-independent functions of the p19(ARF) tumor suppressor. Genes Dev. 2000 Sep 15;14(18):2358–65.

53. Serrano M. The tumor suppressor protein p16INK4a. Exp Cell Res. 1997 Nov 25;237(1):7–13.

54. Guenova M, Rassidakis GZ, Gorgoulis VG, Angelopoulou MK, Siakantaris MR, Kanavaros P, et al. p16INK4A is regularly expressed in Hodgkin's disease: comparison with retinoblastoma, p53 and MDM2 protein status, and the presence of Epstein-Barr virus. Mod Pathol Off J U S Can Acad Pathol Inc. 1999 Nov;12(11):1062–71.

55. Ohtani N, Brennan P, Gaubatz S, Sanij E, Hertzog P, Wolvetang E, et al. Epstein-Barr virus LMP1 blocks p16INK4a–RB pathway by promoting nuclear export of E2F4/5. J Cell Biol. 2003 Jul 21;162(2):173–83.

56. Reuschenbach M, Waterboer T, Wallin K-L, Einenkel J, Dillner J, Hamsikova E, et al. Characterization of humoral immune responses against p16, p53, HPV16 E6 and HPV16 E7 in patients with HPV-associated cancers. Int J Cancer. 2008 Dec 1;123(11):2626–31.

57. Harada H, Nakagawa K, Iwata S, Saito M, Kumon Y, Sakaki S, et al. Restoration of wild-type p16 down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in human gliomas. Cancer Res. 1999 Aug 1;59(15):3783–9.

58. Evangelou K, Bramis J, Peros I, Zacharatos P, Dasiou-Plakida D, Kalogeropoulos N, et al. Electron microscopy evidence that cytoplasmic localization of the p16(INK4A) “nuclear” cyclin-dependent kinase inhibitor (CKI) in tumor cells is specific and not an artifact. A study in non-small cell lung carcinomas. Biotech Histochem Off Publ Biol Stain Comm. 2004 Feb;79(1):5–10.

59. Haller F, Agaimy A, Cameron S, Beyer M, Gunawan B, Happel N, et al. Expression of p16INK4A in gastrointestinal stromal tumours (GISTs): two different forms exist that independently correlate with poor prognosis. Histopathology. 2010 Feb;56(3):305–18.

60. Nilsson K, Landberg G. Subcellular localization, modification and protein complex formation of the cdk-inhibitor p16 in Rb-functional and Rb-inactivated tumor cells. Int J Cancer. 2006 Mar 1;118(5):1120–5.

61. Souza-Rodrígues E, Estanyol JM, Friedrich-Heineken E, Olmedo E, Vera J, Canela N, et al. Proteomic analysis of p16ink4a-binding proteins. Proteomics. 2007 Nov;7(22):4102–11.

62. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol. 2000 Mar;182(3):311–22.

63. Petry KU, Schmidt D, Scherbring S, Luyten A, Reinecke-Lüthge A, Bergeron C, et al. Triaging Pap cytology negative, HPV positive cervical cancer screening results with p16/Ki-67 Dual-stained cytology. Gynecol Oncol. 2011 Jun 1;121(3):505–9.

64. p16/ki-67 dual-stain cytology in the triage of ASCUS and LSIL papanicolaou cytology: results from the European equivocal or mildly abnormal Papanic... - PubMed - NCBI [Internet]. [cited 2017 Jul 28]. Available from: https://www.ncbi.nlm.nih.gov/pubmed/21442767

65. Wentzensen N, Schwartz L, Zuna RE, Smith K, Mathews C, Gold MA, et al. Performance of p16/Ki-67 immunostaining to detect cervical cancer precursors in a colposcopy referral population. Clin Cancer Res Off J Am Assoc Cancer Res. 2012 Aug 1;18(15):4154–62.

66. Prospective evaluation of p16/Ki-67 dual-stained cytology for managing women with abnormal Papanicolaou cytology: PALMS study results - Bergeron - 2015 - Cancer Cytopathology - Wiley Online Library [Internet]. [cited 2017 Jul 28]. Available from: http://onlinelibrary.wiley.com/doi/10.1002/cncy.21542/full

67. Wright TC, Behrens CM, Ranger-Moore J, Rehm S, Sharma A, Stoler MH, et al. Triaging HPV-positive women with p16/Ki-67 dual-stained cytology: Results from a sub-study nested into the ATHENA trial. Gynecol Oncol. 2017 Jan 1;144(1):51–6.

68. Ziemke P. p16/Ki-67 Immunocytochemistry in Gynecological Cytology: Limitations in Practice. Acta Cytol. 2017;61(3):230–6.

69. Piaton E, Advenier AS, Carré C, Decaussin-Petrucci M, Mege-Lechevallier F, Ruffion A. p16(INK4a) /Ki-67 dual labelling as a marker for the presence of high-grade cancer cells or disease progression in urinary cytopathology. Cytopathol Off J Br Soc Clin Cytol. 2013 Oct;24(5):327–34.

70. Piaton E, Carré C, Advenier A-S, Decaussin-Petrucci M, Mège-Lechevallier F, Lantier P, et al. p16INK4a overexpression and p16/Ki-67 dual labeling versus conventional urinary cytology in the evaluation of urothelial carcinoma. Cancer Cytopathol. 2014 Mar 1;122(3):211–20.

71. Yu L-L, Chen W, Lei X-Q, Qin Y, Wu Z-N, Pan Q-J, et al. Evaluation of p16/Ki-67 dual staining in detection of cervical precancer and cancers: a multicenter study in China. Oncotarget. 2016 Mar 23;7(16):21181–9.

72. Waldstrøm M, Christensen RK, Ørnskov D. Evaluation of p16INK4a/Ki-67 dual stain in comparison with an mRNA human papillomavirus test on liquid-based cytology samples with low-grade squamous intraepithelial lesion. Cancer Cytopathol. 2013 Mar 1;121(3):136–45.

73. Shen-Gunther J, Wang C-M, Poage GM, Lin C-L, Perez L, Banks NA, et al. Molecular Pap smear: HPV genotype and DNA methylation of ADCY8, CDH8, and ZNF582 as an integrated biomarker for high-grade cervical cytology. Clin Epigenetics. 2016 Sep 13;8:96.

Appendix

Appendix: 01

Protocol for thin prep cervical smear:

1. PreservCyt solution is a media used for collection of and preservation of cells and DNA of patient samples.
2. All specimens were subjected to standard methods of cell processing.
3. All residual samples were placed inside the refrigerator 2-4 degree Celsius.
4. Thin Prep®) slides prepared on a Thin Prep® 2000 Processor (Hologic™ Inc.) according to the manufacturer's protocol.
5. The residual sample in the container was placed inside the instrument.
6. Cell suspension was homogenized and cells were settled on a filter membrane and transferred to a glass slide.

Appendix:02

Protocol for Hr-HPV DNA test:

1. Label hybridization plate, prepare denaturation reagent.
2. Pipette denaturation reagent (volume is equivalent to half of the specimen volume) into calibrator, quality control and specimen. Check that all tubes show purple colour.
3. Cover rack with film and lid.
4. Vortex for 10 seconds
5. Incubate at 65± 2-degree C for 45± 5 minutes.

6. Prepare HPV probe mix.
7. Microplate heater 1 method used for following steps. Mix denatured specimen well and pipette 75ul into microplate wells.
8. Incubate for 10 minutes at 20-25-degree C
9. Pipette 25ul high risk HPV probe mix into microplate wells.
10. Cover microtubes with a plate sealer and shake on rotary shaker 1 at 1100 ±100 rpm for 3± 2 minutes. Check that all wells show yellow colour.
11. Incubate at 65±2-degree C for 60± 5 minutes. Prepare capture microplate.
12. Transfer contents from each hybridization plate well to corresponding well in capture microtube.
13. Shake at 1100±100 rpm at 20-25-degree C for 60± minutes. Prepare wash buffer.
14. Decant and blot capture microplate.
15. Pipette 75ul detection reagent 1 into each well of capture microplate. Incubate at 20-25-degree C for 30-45 minutes.
16. Manual washing method is used for washing 6 times.
17. Blot on lint-free paper towels.
18. Pipette 75ul detection reagent 2 into each well of capture microplate. Incubate at 20-25-degree C for 15-30 minutes.
19. Read capture microplate on luminometer.
20. Validate assay and interpret specimen results.

Appendix: 03

Protocol for manual staining of CINtec dual immunostaining kit:

Thin Prep slides should be fixed in 100% ethanol for 10 minutes to 1 hour and then air-dried for 20 minutes**.** The kit contains reagent volume which is sufficient to perform 50 tests. 200 µL per slide is recommended to process Thin Prep® cytological preparations.

I. Reagent Preparation

It is recommended to prepare the following reagents, except the Fast-Red working solution, before starting with the staining procedure. All reagents should be equilibrated to room temperature (20 – 25°C) prior to immunostaining.

1. **Epitope Retrieval Solution**

Prepared by dilution of a quantity of Epitope Retrieval Solution 1:10 using distilled or deionized water.

2. **Wash Buffer**

Prepared by diluting of a quantity of the Wash Buffer (10 x) 1:10 using distilled or deionized water.

3. **Substrate-Chromogen Solutions (DAB and Fast Red)**

Chromogens and Substrates are equilibrated to room temperature (20 – 25°C).

A) Preparation of the DAB working solution prior to the staining run

i) One mL of the DAB Substrate Solution into a clean reaction tube;

ii) Add one drop (25 – 30 µL) of the DAB Chromogen and mix gently by inverting the tube (do not vortex)

B) Preparation of the Fast-Red working solution directly before use

Fast Red working solution directly before use. Do not vortex

1. One mL of the Naphthol Phosphate Substrate Solution into a clean reaction tube.

2. Add one drop (40 – 45 µL) of the Fast-Red Chromogen and mix gently by inverting the tube (do not vortex)

4. **Counterstain**

The DAB and Fast Red staining reactions result in water insoluble coloured end product (DAB: brown; Fast Red: red).

Alcohol-free hematoxylin must be used for counterstaining.

5 Mounting Medium.

CINtec® *PLUS* Mount, an aqueous-based permanent mounting medium, is applied in a thin coat and allowed to dry ("liquid cover slipping").

II. **Staining Procedure:**

1**. Specimen rehydration**

For all cytological specimens a rehydration step is necessary prior to staining. This step should be performed at ambient temperature (20 - 25°C). Slides are kept in distilled or deionized water and incubate for 10 (±3) minutes.

2. **Epitope Retrieval**

• Fill heat resistant staining jars (plastic) with the diluted Epitope Retrieval Solution.

• Place staining jars containing Epitope Retrieval Solution in water bath and heat water bath and the Epitope Retrieval Solution to 95 – 99°C.

• When the temperature of 95 – 99°C has been reached immerse the rehydrated cytology slides into the preheated Epitope Retrieval Solution in the staining jars.

• Incubate for 10 (±1) minutes at 95 – 99°C.

• Remove the entire jar with slides from the water bath.

• Remove the lid off the staining jars and allow the slides to cool in the Epitope Retrieval Solution for 20 (±1) minutes at ambient temperature until it has reached 50°C or below.

• Transfer the slides into a staining jar filled with Wash Buffer and incubate for 5 (±1) minutes prior to staining.

3. Steps for staining:

1. Equilibration: Keep the slides with Wash Buffer (2 mL) and incubate for 5 minutes to allow the wash.

2. Apply 200 µL Peroxidase Blocking Reagent. Incubate for 5 minutes.

3. Keep the slides in wash buffer (2 mL) and incubate for 5 minutes.

4. Apply 200 µL Primary Antibodies Solution (p16INK4a/Ki-67). Incubate for 30minutes.

5. Keep the slides in Wash Buffer (2 mL) and incubate for 5 minutes.

6. Apply 200 µL Visualization Reagent HRP. Incubate for 15 minutes.

7. Keep the slides in Wash Buffer (2 mL) and incubate for 5 minutes.

8. Apply 200 µL Visualization Reagent AP. Incubate for 15 minutes;

9. Keep the slides in Wash Buffer (2 mL) and incubate for 5 minutes.

10. Apply 200µL **DAB** Substrate-Chromogen Working Solution and incubate for 10 minutes.

11. Keep the slides with distilled or deionized water (2 mL) and incubate for 5 minutes.

12. Put the slides in Wash Buffer (2 mL) and incubate for 5 minutes.

13. Apply 200 µL **Fast Red** Substrate-Chromogen Solution and incubate for 15 minutes.

14. Keep the slides in Wash Buffer (2 mL) and incubate for 5 minutes.

15. Add 200 µL Alcohol-free hematoxylin must be used for counterstaining for 15 minutes.

16. Permanent mounting medium, is applied in a thin coat and allowed to dry.

Department of Gynecological Oncology, Christian Medical College

Information sheet

You are requested to participate in a study to see the significance of p16/ki67 immunocytochemistry in improving the efficiency of the screening system for cervical cancer in pap smear.

What is pap smear and p16/ki67 immunocytochemistry?

Cervical cancer is a common disease among women in India. Cervical cancer has a high death rate. This cancer is usually screened for by a special test called PAP test. This test uses a spatula to obtain samples from the cervix. These samples are processed into smears which are then subsequently assessed for the presence of cancer cells and categorized according to the risk for cancer as ASCUS/LSIL/ASC-H and HSIL. The categories with the lowest cancer potential include ASCUS and LSIL. It has been found that these two categories despite being regarded as low risk for cancer, also have 5-30% chance of having an underlying cancer. Currently hr-HPV DNA test is used in low grade risk group to identify high grade lesion. If above test is positive patient will be directed for colposcopy. This study proposes to employ special cancer markers (p16/ki67) to help more accurately identify the presence of cancer cells on Pap smears in the lesions. Hence if this study is significant, we can avoid unwanted colposcopy examination and can improve the efficiency of cervical cancer screening system.

What is colposcopy? This is a standard method of evaluating the cervix or entrance to the uterus. It requires standard gynaecological examination using a speculum to see the cervix, A good light and magnification system is used to look at the cervix. During colposcopy diluent vinegar acetic acid will be applied on the cervix and changes in colour noted. If there are abnormal areas, a small biopsy will be taken to confirm cervical pre-cancer or cancer. Even if colposcopy does not show abnormal areas, a 4-quadrant biopsy will still be performed to rule out a precancerous lesion.

Does colposcopy have any side effects? As with any gynaecological examination there may be discomfort and embarrassment of a speculum examination.

If you take part what will you have to do? Colposcopy will be used to look at the cervix. Diluent 3% acetic acid will be applied on the cervix. Abnormal areas may be biopsied. You may have to return and obtain the biopsy result if it has been taken. If biopsy show any abnormality, you will be treated appropriately. It is always better to treat pre- cancer and cancer as early possible.

Can you withdraw from this study after it starts? Your participation in this study is entirely voluntary and you are free to decide to withdraw permission to participate this study.

What will happen if you develop any study related injury? We do not expect any injury to happen to you but if you do develop any side effects or problem due to the study, these will be treated and no cost to you. We are unable to provide any monetary compensation.

Will you have to pay for the study? Colposcopy will be done without charge. You need to pay for biopsy.

What happens after the study is over ?We will be able to know whether p16/ki67 dual immunostaining will be useful in improving the efficiency of the screening system for cervical cancer. So that in the future, it will be recommended in abnormal pap test.

Will your personal details be kept confidential? The results of this study will be published in medical journal but you will not be identified by name in any publication or presentation of results. However, your medical data noted may be reviewed by people associated with the study without your additional permission.

If you have any further questions, please ask.

Dr. Vinoth Kumar (9952541163) E-mail**:** **rogervinoth@gmail.com.**

Informed Consent Form for Subjects

Study Title: To assess the significance of p16/ki67 immunocytochemistry in improving the efficiency of the screening system for cervical cancer.

Study Number: _____ Subject's Name: _____

Date of Birth / Age: _____

(i) I confirm that I have read and understood the information sheet dated _____for the above study and have had the opportunity to ask questions. [ ]

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

(iii) I understand that, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). [ ]

(v) I agree to take part in the above study. [ ]

Signature (or Thumb impression) of the Subject/Legally Acceptable

Date: _____/_____/_____

Signatory's Name: _____ Signature:

Clinical Research Form

- **Title: To improve the predictive value to identify CIN 2+ lesion in cervical pap smears by using novel p16/ki67 immunocytochemistry test.**

- SI No: Date:
- Name:
- Age:
- Hospital No:
- Cytology No:
- Histopathology- Biopsy No:
- Indication for cervical screening: Routine cervical screening / Symptomatic
- Presenting symptoms: Irregular bleeding per vaginum / Discharge Per vaginum / Growth / Ulcer.
- Duration of illness:
- Examination findings:
- Marital status: Married / Unmarried
- Menstrual status:
- Menopause – Yes/No
- If yes, since how many years?

Step - 1: Cervical pap smear screening: Negative/ reactive change/ ASC-US/ LSIL / ASC-H/ HSIL/ AGC/ SCC/ Adenocarcinoma.

Step-2: hr-HPV DNA test: Positive / Negative.

Step-3: Dual immunostaining p16/ki67: Positive/Negative.

Step-4: Colposcpy examination: Satisfactory / Positive / Negative.

Step-5: Biopsy in colposcopy positive cases: Negative/ Reactive changes/ CIN1 /CIN11 / CIN111 / Invasive malignancy.

SI.NO	hr-HPV	p16/ki67 dual immunocytochemistry	Colposcopy	Biopsy

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

September 17, 2016

Dr. Vinoth Kumar. G,
PG Registrar,
Department of Pathology,
Christian Medical College,
Vellore 632 004.

Sub: **Fluid Research Funding: New Proposal**
To assess the significance of p16/ki67 immuno cytochemistry in improving the efficiency of the screening system for cervical cancer.
Dr. Vinoth Kumar. G, PG registrar, general pathology, Dr. Anne Jennifer Prabhu (Employment Number: 28224.), General Pathology, Dr. Abraham Peedicayil, Employment number: 03024, Gyneoncology, Dr. Priya Abraham, Employment number: 11714, Virology, Mr. A. Raghavendran Employment number: 32370, Virology.

Ref: IRB Min No: 10175 [OBSERVE] dated 06.07.2016

Dear Dr. Vinoth Kumar. G,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Biju George, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS., MD., DM.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Anne Jennifer Prabhu, Dept. of Pathology, CMC, Vellore

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. Sathish	MBBS, MD, DCH	Professor, Child Health, CMC, Vellore	Internal, Clinician
Dr. Rekha Pai	MSc, P.hd	Internal Basic Scientist, Internal Basic Scientist, CMC, Vellore	External, Legal Expert
Dr. Thomas V Paul	MBBS, MD, DNB, PhD	Professor, Endocrinology, CMC, Vellore	Internal, Clinician
Dr. Rajesh Kannangai	MD, PhD.	Professor, Clinical Virology, CMC, Vellore	Internal, Clinician
Dr. Ranjith K Moorthy	MBBS, MCh	Professor, Neurological Sciences, CMC, Vellore	Internal, Clinician
Dr. Santhanam Sridhar	MBBS, DCH, DNB	Professor, Neonatology, CMC, Vellore	Internal, Clinician

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of withdrawals for the study entitled: "To assess the significance of p16/ki67 immuno cyto chemistry in improving the efficiency of the screening system for cervical cancer" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in).

Fluid Grant Allocation:
A sum of 1,00,000/- INR (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 50,000/- INR (Rupees Fifty Thousand only) each will be released at the end of the first year as 2 nd Installment.

Yours sincerely,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS., MD., DM.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

IRB Min No: 10175 [OBSERVE] dated 06.07.2016

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

Data Sheet

slno	age	cytology	biopsyno	cervical	presentsym	durillness	marital	parity	menopause	menoyes	papsmear	dnatest	dualimmuno	ifposcells	cell	colposcpy	biopcolp
1	60	P16-13089	32402/6	1			1	3	1	7	4	1	1	3	3	2	7
2	61	P16-14508	36058/16	2	5		1	3	1	4	1	2	2		2	2	3
3	30	P16-13495	37017-17	2	2	30	1	3	2		1	2	2		2	2	1
4	47	P16-14523	36042/16	2	1	2	1	3	2		1	2	2		3		5
5	46	P16-15561	38187/16	2	1	18	1	3	2		1	2	2			1	5
6	45	P16-15888	38362/16	2	3		1	2	2		1	1	2		3		3
7	26	P16-15453	39363-16	2	1		1	3	2		4	1	1	2	3	3	3
8	57	P16-15804	39261/61	1			1	2	1	10	3	1	1	2	3	1	5
9	49	P16/15432	39265/16	1			1	3	1	2	1	2	1	2	3	1	3
10	29	P16-14395	39267/16	2	5		1	3	2		1	2	1	2	3	1	3
11	54	P16-16012	38851/16	2	1	12	1	3	2		2	1	1	3	3	2	6
12	60	P16-14713	39427/16	2	5		1	3	1	10	1	2	2		3	1	2
13	34	P16-16003	39774/16	2	1	6	1	3	2		1	2	2		3		3
14	42	P16-16394	39792-16	2	2	24	1	2	2		1	2	2		3		3
15	29	P16/16005	39853/16	2		5	1	1	2		1	2	2		3		3
16	37	P16-16407	39818/16	2	1	48	1	3	2		4	1	1	3	3	2	6
17	36	P16-15729	39970/16	2	1	12	1	3	2		2	1	2		3	1	3
18	35	P16-15899	39986/16	2	1	36	1	2	2		1	2	2		3		3
19	70	P16-16856	40742/16	2	1	1	1	3	1	25	4	1	1	3	3	2	8
20	23	P16-16824	41021/16	2	5		1	3	2		3	1	1	2	3	1	5
21	52	P16-16906	41139/16	2	1	0.12	1	3	1	2	1	2	2		3	1	3
22	45	P16-17402	42082/16	2	1	60	1	3	2		4	1	1	3	3	2	7
23	51	P16-16544	41700/16	2	5	24	1	3	1	1	3	1	1	3	3	1	6
24	33	P16-17603	42593/16	2	5	36	1	3	2		1	2	2		3	1	3
25	27	P16-17522	42730/16	2			1	2	2		2	1	1	2	3	3	3
26	40	P16-15496	15496/16	2	1		1	3	2		1	1	1	3	3		6
27	26	P16-17882	4448/16	1			1	3	2		1	2	2		3	1	3
28	61	P16-18306	44436/16	1			1	3	1	6	1	1	2		3	1	3
29	45	P16-18773	44855/16	2	5	3	1	3	2		3	2	2		3	1	4
30	42	P16-18133	48864/16	2	6	6	1	3	2		1	2	2		3	1	3
31	33	P16-18388	4956/17	2	1	12	1	3	2		4	1	1	3	3	2	7
32	42	P16-19095	45073/16	2	1	60	1	3	2		1	2	2		3	2	2
33	41	P16-19453	46380/16	2	1	4	1	3	2		1	2	2		3	3	2
34	45	P16-20443	39/17	2	1	4	1	3	2		4	1	1	2	3		8
35	36	P16-20285	639/17	2	1	1	1	2	2		1	2	2		3	1	3
36	47	P17/2067	1061/17	2	1	72	1	3	2		4	1	1	3	3	3	6
37	31	P17-152	1605/17	1					2		2	1	2		3		4
38	40	P17-643	2870/17	2	1		1	2	2		4	1	1	3	3	2	6
39	63	P17-1280	2481/17	2	1		1	3	1	7	4	1	1	3	3		8
40	72	P17-1743	5226/17	2	1	0.15	1	3	1	15	1	2	2		3	3	3
41	26	P16-7882	44448/16	1			1	3	2		1	2	2		3	1	3
42	47	P16-19680	46998/16	2	2	2	1	3	1	3	4	1	1	3	3	2	8
43	36	P16-19398	46992/16	2	2	0.4	1	3	2		4	1	1	3	3	2	7
44	70	P16-19526	47000/16	1			1	3	1	26	1	1	1	2	3	2	6
45	43	P16-19620	47371/16	2	1	1	1	3	2		1	1	1	3	3	2	3
46	43	P16-14512	35464/16	2	1		1	3	2		1	2	2		2		3
47	40	P16/18039	47388/16	2	1	2	1	3	2		2	1	1	2	3	1	5
48	28	P16-20088	47946/16	2	2		1	3	2		3	1	1	3	3	2	6
49	36	P17-1026	207/17	2	1		1	3	2		2	1	2		3	2	4
50	62	P16-20431	203/17	2	7		1	3	1	12	1	2	2		3	2	5

51	35	P16-20285	639/17	2	5		1	3	2		1	2	2		3	3	3
52	32	P17-337	17-337	1			1		2		1	1	2		3	2	3
53	47	P16-20671	1060/17	2	1	72	1	3	2		4	1	1	3	3		6
54	21	P17-544	41/17	1			1	3	2		3	1	1	2	3	2	4
55	58	P16-20493	7339/17	1			1	3	1	10	1	1	1	3	3	2	6
56	49	P17-1219	4260/17	2	5		1	3	1	4	3	1	1	2	3	2	5
57	46	P17-1519	4547/17	1			1	2	1	1	1	2	2		2	2	3
58	41	P16-19012	4292/16	2	5	1	1	2	2		1	1	2		3	2	3
59	39	P16-15851	38514/16	1			1	2	2		3	2	2		3	2	3
60	36	P17-2431	5662/17	2	1	0.5	1	3	2		2	1	2		3	1	3
61	36	P17-2249	7631/17	2	1	1	1	2	2		1	1	2		3	2	4
62	46	P17-2725	7579/17	2	1	4	1	3	2		4	1	1	2	3	3	7
63	27	P17-3417	8359/17	2	1		1	2	2		2	1	1		3	2	5
64	39	P17-3019	7628/17	1			1	3	2		3	1	1	3	2	2	2
65	48	P17-1774	4700/17	1	1	2	1	3	2		2	2	2		3	2	5
66	52	P17-585	4694/17		5		1	3	2		3	1	1	3	3		7
67	40	P17-1772	4704/17	2	2	2	1	1	2		1	1	2		3	1	5
68	45	P17-3840	12058/17	2			1	3	2		2	1	1	2	3	1	3
69	40	P17-3962	9482/17	2	1	12	1	2	2		3	2	1	2	3	2	3
70	44	P16-20527	6212/17	2	2	12	1	3	2		1	1	1	2	3	1	5
71	37	P16-16609	41016/16	1			1	2	2		2	1	1	2	3	1	4
72	24	P16-19438	5231/17	2	1	3	1	3	2		1	1	1	3	3	1	3
73	39	P16-19524	4549/17	2	1	3	1	3	2		2	2	2		3	2	3
74	53	P17-5465	13052/17	2			1	3	1	4	4	1	1	3	3	2	7
75	36	917-970	13060/17	2	6		1	3	1	15	1	1	1	2	3		3
76	47	P17-3699	12113/17	2	2	6	1	3	2		4	1	1	3	3	1	8
77	45	P17-3028	6612/17	2	1	2	1	3	2		3	1	1	2	3	2	6
78	43	P17/1042	4698/17	2	2	6	1	3	2		1	2	2		3	2	4
79	40	P17/6610	14850/17	2	1	7	1	3	2		4	1	1	3	3		8
80	44	P16-20620	47212/16	2	1	2	1	3	2		1	2	1	2	3		2
81	34	P16-21993	28819/16	2	2		1	3	2		2	1	2		3	2	3
82	53	P16/2024	2774/16						1	1	4	1	1	3	3		7
83	43	P16-19452	44387/16	2	1	4	1	3	2		4	1	1	3	3		7
84	42	P16-2410	11596/16	2	1	24	1	3	2		1	2	2		3		3
85	60	P16-126	280/16	2	1	12	1	3	1	8	4	1	1	3	3	2	7
86	64	P16-19132	2391/16	2			1	3	1	15	2	1	1	3	3	2	6
87	41	P16-501	2147/16	2	2	60	1	3	2		3	2	2		3		6
88	41	P16-16612	40247/16	2	1	2	1	3	2		1	2	2		2		2
89	65	P16-15933	40596/16	2	5	3	1	3	1	20	4	1	1	3	2		8
90	34	P17-2216	5478/17	2	6		1	2	2		2	2	2		2		3
91	63	P16-19392	46382/16	1			1	3	1	10	1	1	2		2		3
92	59	P17-657	2009/17	2	5				1	3	4	1	1	2	2		7
93	31	P16-18815	46155/16	2	1	5	1	3	2		2	1	2		3		3
94	31	P17-445	13560/17	2	1	5	1	3	2		3	1	2		2		3